UNIVERSITY OF ALBERTA

The Characterization of a Novel, Widespread, PNA-Reactive Tumor Associated Antigen: The Alpha-fetoprotein Receptor/Binding Protein

by

Marian P. Laderoute

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled *The Characterization of a Novel, Widespread, PNA-Reactive Tumor Associated Antigen: The Alpha-fetoprotein Receptor/Binding Protein* submitted by Marian P. Laderoute in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Medical Sciences (Immunology).

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Dedication

This thesis is dedicated to Dr. G.I. Abelev (Moscow) who first reported the discovery of the alpha-migrating fetal protein (alpha-fetoprotein) in tumors in 1963.

Key Words

Alpha-fetoprotein Receptor, Alpha-fetoprotein, Alpha-fetoprotein Binding Protein, Thomsen-Friedenreich Antigen, TF Antigen, Tn Antigen, PNA-Reactive Tumor Antigens, Oncofetal Antigens, Non-specific Immunosuppressive Factors, Breast Cancer Prognostic Markers, Immune Surveillance, Tumor Progression Factors, Oncogenesis, Cellular Senescence, Anti-cellular Senescence Oncogenes, Programmed Cell Death, Thymocyte Development, Tumor Immunology.

ABSTRACT

Accumulating evidence has suggested that Peanut Agglutinin (PNA) reactive tumor associated antigens are expressed in 90 % of common human adenocarcinomas and might play an important role in human malignancy. In order to characterize and elucidate the potential role of these tumor associated antigens, monoclonal antibodies (MAbs) were made to PNA affinity enriched glycoproteins extracted from pooled breast cancer biopsy membrane materials. Two of the resulting MAbs, the 167H.1 and 167H.4 MAbs, were further investigated as they strongly reacted with antigens expressed in approximately 90 % of common human adenocarcinomas as assessed by the immunoperoxidase technique on frozen sections. The antigen recognized by both MAbs was subsequently identified as an alpha-fetoprotein binding protein (AFP-BP) by the ability of these MAbs to specifically inhibit the binding of AFP to cell surfaces, by the ability of alpha-fetoprotein (AFP) to specifically block the binding of the MAbs to soluble sources of the AFP-BP, and by the purification of the AFP-BP on the basis that it could specifically bind AFP. Western blotting, immunoprecipitation, gel filtration and analysis of the purified AFP-BP indicated that this soluble and membrane associated antigen has a molecular weight of approximately 67 kilodaltons (kd). The PNA reactivity of the AFP-BP was confirmed on purified antigen by specific carbohydrate inhibition testing.

The molecular nature of the cell surface AFP receptor was not completely elucidated although preliminary evidence suggested that the 67 kd AFP-BP may associate with a 185 kd molecule which may become phosphorylated upon stimulation of the former with the 167H.1 MAb. The 167H.1 and 167H.4 MAbs have biological activity and react with cell surfaces implying that the AFP-BP or a cross-reactive antigen forms part of the functional AFP receptor. Whether the 67 kd antigen contains a transmembrane region remains to be determined by sequence information, however.

The 167H.1 and 167H.4 MAbs were found to behave as AFP agonists and in agreement with previously published findings, the results suggested that AFP may abrogate cellular induction signals involved in proliferation and/or differentiation of normal and

malignant cells. These effects were extended to include the abrogation of cellular senescence in experiments with malignant cells (HL-60) and with normal cells (developing multinegative human thymocytes) as evidenced by the prevention of programmed cell death. Of particular relevance to oncogenesis, the use of these MAbs has confirmed that AFP is an immunosuppressive molecule. It is proposed that AFP and AFP binding proteins may play an important autocrine role in human oncogenesis by their ability to abrogate cellular senescence of tumor cells. This may contribute to the immortality of tumor cells and/or provide resistence of tumor cells to induction of immune-mediated apoptosis. An important paracrine role of these antigens may relate to the inhibition of host immunological defence mechanisms which may render the tumor more malignant.

Overall, this appears to be the first characterization of a PNA-reactive tumor associated antigen which is expressed in 90 % of adenocarcinomas, may be the first elucidation of its biological importance in oncogenesis, and may be the first documentation of its potential prognostic significance. Finally, this may be the first conclusive identification and characterization of the AFP binding protein/receptor.

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Studies implicating that the novel tumor associated antigen described herein may be the elusive alpha-fetoprotein binding receptor were initiated in collaboration with Dr. Ricardo Moro. Dr. William McBlain of the Hormone Receptor Laboratory, provided breast cancer biopsy membranes and cytosols for analysis as well as estrogen and progesterone receptor levels for the blinded study on cytosols. FPLC studies were performed under the guidance of Ewa Pruski. The gel filtration by HPLC of the semi-purified AFP-BP, was performed by Michael Voralia. A special thanks to Hossam Sharkawi and Ewa Pruski for help in performing FACS analysis and again to Ewa for the isolation and depletions of human thymocyte populations. Much thanks to Diane Swanlund for the work performed with the synthetic TF and Tn antigens. Dr. Simeon Vassiliadis provided additional work on the enhancement of HL-60 proliferation by AFP and the 167H.1 MAb. The immunohistological studies were performed at the Edmonton General Hospital under the direction and interpretation of Dr. Dave Willans and all are gratefully acknowledged. I would also like to acknowledge the warmth and guidance of my co-workers, Bobina, Mila, Vasek, Ewa, Rucy and Terry and the support and friendship of the many people I have come to know over the past several years but particularly: Ann Burrell, Pam Eden, Rose Saponja, Dr. Santanu Das, Drs. Rati and Arun Fotedar, Dr. Vincent LaPosta, Don Branch et al, JoAnn Mackie, Dr. Vassili Karanassios, Dr. Gitte Jensen, Dr. Maureen O'Connor-McCourt, Dr. Andrzej Greglewski, and last but by no means least, my friend, mentor and colleague, Dr. E.J. Sarcione. I would also like to thank my family for their assistance and generosity through this lengthy ordeal. I would also like to commend my commitee members for their suggestions, advice and concern, but also the invaluable contribution of and support of Dr. Douglas Green in years past. I thank Dr. Michael Longenecker for providing the opportunity for me to carry out most of this research. He deserves an applause not only for his encouragement, enthusiasm and expertise, but his patience which I sometimes feel I taxed quite often. I graciously thank the Alberta Heritage Foundation for Medical Research for their financial support and their Annual Heritage Days Meetings through which I have made some very valuable contacts and at which some of the

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List of Abbreviations

2ME	-B-2-mercaptoethanol
AATM	-AFP-BP Anchoring and Transducing Molecule
AB serum	-serum from an AB donor
ABTS	-2,2'-azino-di-[3-ethyl]-benzthiazoline sulphonate
ADCC	-antibody dependent cellular cytotoxicity
AFP	-alpha-fetoprotein
AFP-BP	-alpha-fetoprotein binding protein
AFPr	-alpha-fetoprotein receptor (cell surface)
AIDS	-acquired immunodeficiency syndrome
AMF	-autocrine motility factor
APRT	-adenosine phosphoribosyltransferase
ATP	-adenosine tri-phosphate
B cells	-bone marrow derived lymphocytes
BCG	-Bacillus Calmette-Guerin
BCIP	-5-bromo-4-chloro-3-indolyl phosphate-toluidine salt
BSA	-bovine serum albumin
cDNA	-cloned deoxyribonucleic acid
CEA	-carcinoembryonic antigen
c-erbB-1	-EGF receptor
c-erbB-2	-HER-2/neu tyrosine kinase
CSF-1	-colony stimulating factor, one
CNBr	-cyanogen bromide
CON-A	-concanavalin A
c-onc	-cellular (proto)oncogene
cpm	-counts per minute
DAB	-3',3'-diaminobenzidine
ddH_20	-double distilled water

D-gal	-D-galactose
D-glu	-D-glucose
DMF	-N,N-dimethylformamide
DMSO	-dimethyl sulfoxide
DNA	-deoxyribonucleic acid
DTH	-delayed type hypersensitivity
EDTA	-ethylenediaminetetraacetic acid
EGF	-epidermal growth factor
EGFr	-epidermal growth factor receptor
ELISA	-enzyme linked immunosorbent assay
epi	-epiglycanin, a murine tumor TF antigen
ER	-estrogen receptor
FACS	-fluorescence activated cell sorting
FCS	-fetal calf serum
FITC	-fluorescein isothiocyanate
FL1	-FITC fluorescence by FACSCAN analysis
FPLC	-fast pressure liquid chromatography
FSC	-forward scatter (size parameter)
g	-grams
G	-gravity
gal	-D-galactose
galNAc	-N-acetyl-galactosamine
GBSS	-Gey's Balanced Salt Solution (with Mg/Ca^{++})
gluNAc	-N-acetyl-glucosamine
gel	-gelatin
H_2O_2	-hydrogen peroxide
HAFP	-human AFP
HCC	-hepatocellular carcinoma

HCl	-hydrochloric acid
HPA	-*Helix pomatia* lectin
HPLC	-high pressure liquid chromatography
HPRT	-hypoxanthine phosphoribosyltransferase
HRP	-horse-radish peroxidase
HSA	-human serum albumin
HTP	-hydroxylapatite
Ia	-I region encoded proteins of MHC, Class II antigens
IEF	-isoelectric focussing
IFN	-interferon
Ig	-immunoglobulin
IL-2	-interleukin 2
IMDM	-minimal essential media
i.p.	-intraperitoneal
IPTG	-isopropyl-B-D-thiogalactopyranoside
kb	-kilobases
KCl	-potassium chloride
kd	-kilodalton
K_d	-dissociation constant
KLH	-keyhole limpet hemocyanin
LAK	-lymphokine activated killers
LB	-liquid broth
L-CM	-L-cell conditioned medium
LDL	-low density lipoprotein
LPS	-lipopolysaccharide
M	-molar
mCi	-millicurie (10^{-3} curies)
mM	-millimolar (10^{-3} molar)

MAb	-monoclonal antibody
MEM	-minimal essential media
mg	-milligram (10^{-3} gram)
$MgCl_2$	-magnesium chloride
$MgCl_2 2\text{-}6H_2O$	-magnesium chloride
MHC	-major histocompatibility complex
ml	-millilitre (10^{-3} litre)
MLR	-mixed lymphocyte reaction
MMTV	-Mouse Mammary Tumor Virus
MPa	-milli-pascals
mRNA	-messenger ribonucleic acid
MSA	-mouse serum albumin
MTT	-(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
N	-normal
Na_2HPO_4	-sodium phosphate, dibasic
$Na_2HPO_4 \cdot 7H_2O$	-sodium phosphate, dibasic
$NaBH_4$	-sodium borohydride
NAbs	-naturally occurring antibodies
NaCl	-sodium chloride
NaH_2PO_4	-sodium phosphate
$NaHCO_3$	-sodium bicarbonate
Nana	-n-acetyl-neuraminic acid (sialic acid)
NaOH	-sodium hydroxide
N'ase	-neuraminidase treated
NBT	-p-nitro blue tetrazolium chloride
ND	-not done
NH_4Cl	-ammonium chloride
NK	-natural killer

nm	-nanometers (10^{-9} meters)
NMS	-normal mouse serum
NP-40	-nonidet P-40
NS	-natural suppressor cells
OA	-ovalbumin
O.D.	-optical density
OFA	-oncofetal antigen
PBMC	-peripheral blood mononuclear cells
PBS	-phosphate buffered saline (no $Mg/Ca^{+/+}$)
PCD	-programmed cell death
PDGF	-platelet derived growth factor
PE	-see PTNT 89
PgR	-progesterone receptor
pH	-log (H+)
PHA	-phytohaemagglutinin
pI	-isoelectric point
PI	-propidium iodide
PMSF	-phenylmethyl sulfonyl fluoride
PNA	-Peanut Agglutinin lectin
PTNT 89	-a pleural effusion of a lung metastatic breast carcinoma
RBC's	-red blood cells (human)
RER	-rough endoplasmic reticulum
rpm	-revolutions per minute
RPMI	-Roswell Park Memorial Institute media
s.d.	-standard deviation
SDS	-sodium dodecyl sulphate
SDS-PAGE	-SDS-polyacrylamide gel electrophoresis
s.e.	-standard error

SSC	-side scatter (granularity parameter)
sup	-supernatant
TAA	-tumor associated antigen
Tbm	-NP-40 extracts of tumor breast biopsy membranes
TCA	-tricloroacetic acid
T cell	-thymus derived lymphocyte
TcR	-T cell (antigen) receptor
T-CTL	-cytotoxic T cell
T-DTH	-delayed type hypersensitivity T cell
TF	-Thomsen-Friedenreich
TGF-A	-Transforming Growth Factor Alpha
TGF-B	-Transforming Growth Factor Beta
Tn	-precursor of TF antigen
TNF	-tumor necrosis factor
Ts	-suppressor T cell
u	-units
ug	-microgram (10^{-6} gram)
ul	-microlitre (10^{-6} litre)
v-*onc*	-viral oncogene
VVA	-*Vicia villosa* lectin

I. LITERATURE REVIEW

"Conceivably the process by which malignancy is conferred on the cell automatically enforces a changed antigenic quality on the cell membrane."

F.M. Burnet, 1970.

A. INTRODUCTION

In Alberta the number of deaths due to cancer (1.37 per one thousand per year), is second only to diseases of the heart (Vital Statistics Annual Review, 1987). The number of new cases per year in Canada increased steadily by about 40 per cent in ten years (Canadian Cancer Statistics, 1987). Due to the prevalence of cancer and its expected gain not only in incidence but cost to the health care system, there is a great impetus for the prevention, early diagnosis and cure of human malignancies.

In order to meet these objectives, the onus is to understand how and why cancers develop. An initial transformation event is necessary but not sufficient for the appearance of clinically defined cancer (Sirica, 1989). Thus, a distinction between transformation and oncogenesis must be made. In recent years, our knowledge about transformation has greatly expanded, largely due to the discovery of several dozen or more oncogenes, and to studies of gene products of viruses with transforming potential, such as SV40. In contrast to transformation however, few gene products have been identified which play a role in oncogenesis or tumor progression (Sirica, 1989). The critical question is not what causes or maintains the transformed state, but what permits tumorigenesis and contributes to the malignant potential of tumors in the intact host.

In this treatise the notion that the immune system plays an intimate role in the oncogenic outcome of transformation is first explored. Inherent in this scheme is that there is specificity of recognition of tumor cells by the immune system. Hence, this necessitates a general discussion on the nature of and classification schemes of tumor antigens. Tumor antigens, where a defined biological function have been elucidated, are then briefly discussed. Greater detail is then given on the history and characterization of the tumor associated

antigens which are particularly relevant to this thesis, the Thomsen-Friedenreich (TF) antigen, alpha-fetoprotein (AFP), and the alpha-fetoprotein receptor and binding protein. The literature review then culminates in the raison d'etre of this thesis and research: the hypothesis, the objectives and the experimental approach taken.

B. TRANSFORMATION VERSUS ONCOGENESIS: A ROLE FOR THE IMMUNE SYSTEM

The rationale for the involvement of the immune system in tumorigenesis stems from several lines of evidence.

The realm of viral transformation has provided ample and concrete evidence for the importance of the immune response in tumor progression. Lewis et al (1985) have emphasized that the host's immune response to DNA-virus transformed cells is the critical element in determining the fatality of the neoplasm. Tumor incidence for DNA-virally transformed cells has been inversely correlated to the ontogeny of immuno-competence. For example, neonatal woodchucks infected with the hepatitis B virus and raised in a carcinogen free environment all develop hepatocellular carcinoma (Gerin et al, 1986). Similarily, SV40 transgenic mice that develop tumors are specifically tolerant to the SV40 T antigen (Faas et al, 1987). These experimental animal models suggest that neonatal tolerance increases the incidence of tumor occurrence by viral transformation. However it is not clear if the important factor is the diminished elimination of infective, transforming virus or the decreased ability to reject the tumors which result. These findings however are similar to the clinical findings in hepatocellular carcinoma.

Hepatocellular carcinoma (HCC) is one of the most prevalent types of cancer worldwide with over one quarter of a million people per year who develop and die from this cancer (Kew, 1986). In certain populations in Africa and Asia, HCC is the most common cancer (Kew, 1986, Harrison, 1986). In these populations the endemic hepatitis B virus is the most important causal association. In over 80 per cent of these tumors, integrated viral DNA sequences can be demonstrated (Harrison, 1986). An important factor in the etiological

association between hepatitis B virus and primary liver cancer is the high perinatal transmission of the virus, leading to the carrier state. It has been estimated that 200 million people worldwide are carriers of the hepatitis B virus (Harrison, 1986). There is a 200 - 300 fold risk of developing hepatocellular carcinoma for carriers compared to non-carriers (Harrison, 1986, Kew, 1986). It may be that inability to recognize viral antigens as foreign, allows tumor escape.

Apart from neonatal tolerance schemes involved in virally-transformed tumors, other mechanisms which permit the growth of these tumors in an intact host also involve the inability of the immune system to recognize these tumors as foreign. It is well established that the cellular immune response of cytotoxic T cells (CTL's) requires MHC co-recognition of the Class I type (Zinkernagel, 1976). Diminished Class I expression on tumors correlates to tumorigenesis of virally transformed cells (Garrido et al, 1986, Eisenbach et al, 1984, Brown et al, 1988). For example, the highly oncogenic adenovirus type 12 inhibits Class I expression on virally transformed cells compared to the non-oncogenic adenovirus type 5 (Vaessen et al, 1986, Hayaski et al, 1985, Burgert et al, 1985, Cook et al, 1983). It has been shown that the expression of the E19 gene product present only in the type 12 adenovirus, inhibits the cell surface expression of Class I since it binds to these molecules causing abrogation of terminal glycosylation and lack of cell surface expression (Andersson et al, 1985). Transfection studies have established that this single gene product is required for oncogenesis. This is the first clear example of an oncogenic gene product distinct from those which contribute to transformation. Importantly, it also supports the role of the immune system in tumor progression and/or malignancy in the intact host. More recently, it has been shown that viral transformation of cells is associated with the ability of viral gene products such as E1A to bind to (and thus inactivate) the retinoblastoma gene product which is a known anti-oncogene (Egan et al, 1989). Whether this contributes to oncogeneic transformation or not needs to be established.

Tumors in which a viral etiology cannot be established also show a relationship of tumorigenesis with the downregulation of cell surface Class I expression. *N-myc* transfected neuroblastoma cells (amplified *n-myc*) downregulates Class I expression and this correlates to

tumorigenesis (Bernards et al, 1986). Loss of tumorigenicity of spontaneous lung carcinomas has been correlated to the expression of Class I antigens as studied by transfection experiments (Bahler et al, 1987). Others have shown a similar role of Class I antigens in tumorigenicity for chemically induced fibrosarcomas (Hammerling et al, 1986). These authors also suggest that about 10 - 15 per cent of human or animal tumors are Class I deficient.

Although these results imply a role of the CTL's in tumor rejection, it is clear that these are not the only cells which participate. Other cells which do not rely on co-recognition of Class I, are as important, if not more so in certain systems. These cells comprise the innate immune arm and are made up of macrophages, natural killers (NK) and lymphokine activated killers (LAK) (Barlozzari et al, 1985, Herberman et al, 1989, Kanar et al, 1988). These cells may also mediate antibody-dependent-cytotoxicity (ADCC) which also plays a role in the rejection mechanism. Finally, other cells which appear to co-recognize foreign entities in the context of MHC Class II also exist and have been identified as T cells which mediate delayed type hypersensitivity or T-DTH cells. Thus it is not surprising that Class I expression on tumors may not always correlate inversely to tumor survival. Indeed, Karre et al (1986) have shown a positive correlation of Class I expression to tumor growth for T lymphoma cells. This was explained by a concomitant increased resistence to lysis by NK cells. Others have demonstrated a negative correlation of Class II expression with tumor progression (Powell et al, 1987). Thus, due to the complexity of immune surveillance which involves both innate and adaptive immune cells, it may be an oversimplification to expect MHC expression to predict oncogenic outcome in all instances. However, its involvement in tumor progression substantiates a role of the immune system in oncogenesis.

A stronger argument pertains to the finding that transformed cells usually do not form tumors unless transplanted into immunocompromised hosts (Lewis et al, 1985, Fuji et al, 1986) which provides an obvious distinction between transformation and oncogenesis. This clearly lends credence to the immunological surveillance hypothesis, originally proposed by Thomas in 1959 and extended by Burnet (1970). Although technically, the immune surveillance hypothesis (reviewed in Smith et al, 1970), was originally proposed to explain the

phylogenetic evolution of the adaptive immune response, modern reinterpretation of host resistance to cancers would have to include the innate immune arsenal for protection against the appearance and/or in the rejection of cancers. In this thesis, the use of the term immune surveillance will include the innate as well as the adaptive arms of the immune system. A major argument supporting the immune surveillance hypothesis is that between the ages of 10 and 40, the incidence of cancer is relatively rare, but after 40 associated with the decline of the functioning of the immune system, there is a corresponding increase in the incidence of cancer.

Stutman (1975) argues that immunological mechanisms at least those of the adaptive type, appear to play a surveillance role in only a minority of tumors such as those induced by viruses or high doses of chemical carcinogens. It was not appreciated in 1975 that viruses do appear to cause a high proportion of cancers, (ie. HCC in endemic areas). As Nelson et al (1987) have pointed out, it may be very inappropriate to discount the innate or natural immune arsenal when one attempts to test the immune surveillance hypothesis.

The problem with trying to test the immune surveillance hypothesis in its entirety is that the lack of macrophages is not compatible with life (Fidler et al, 1986, Fidler, 1985). However, for immunosuppressed individuals such as transplant recipients or AIDS patients, there is an increased risk for certain cancers such as lymphomas (Ashman, 1987), or Kaposki's sarcoma (Levine et al, 1987), respectively. There may be a connection between these two as retroviruses induce sarcomas and hematolymphopoietic tumors (Farber, 1986); these patients may not live long enough to develope other types of cancers. Nevertheless, opponents of the immune surveillance hypothesis would argue that these are unusual cancers and thus, cannot explain the situation for the more common types. On the other hand, one might argue that as mentioned above, a complete absence of immunity is never achieved and thus to conclude that immunosuppressed individuals do not exhibit a higher incidence of usual cancers, may be misleading. It may be that AIDS patients die from infection before tumors surface or that retrovirally-caused tumors are the first to surface as this transforming virus is endemic in the AIDS population. Similarly some immunity must exist for transplant patients

as they do not generally succumb to opportunistic infections although infections often afflict these patients. Thus, the argument that immunosuppressed individuals do not succumb to the usual cancers is an oversimplification given the evidence that some residual immunity remains.

The question still emerges as to whether the innate and adaptive arms of the immune response play a role in tumorigenesis and if they do, how might tumor cells escape the immune system? As reviewed in Nelson et al (1987), tumor cells may foil the immune response by mechanisms known as antigen shedding, sneaking through and/or tolerance induction, molecular mimicry, or by generating blocking antibodies. Other mechanisms by which tumor cells may appear to be non-immunogenic include alterations in MHC antigen expression or a special case where the tumor is masked in a protective glycocalyx. This last mechanism seems to be particularly effective as the expression of epiglycanin on the TA3-Ha mammary adenocarcinoma cell line is associated with allo and xenographic growth (Van den Eijden et al, 1979). Its expression is also associated with the malignant growth characteristics of the TA3-Ha in the syngeneic host compared to the strain specific growth of TA3-ST in which the survival times are significantly longer (Miller et al, 1977). However, a more general explanation which may serve to encompass most of the aforementioned possibilities is that tumor progression might be associated with the secretion of immunosuppressive factors. This would be expected to create a local immunosuppressive environment but would be associated with systemic immunosuppression/deregulation in the late stages.

Consistent with this hypothesis is the observation that some human cancers are associated with systemic suppression, such as glioblastomas where after surgical removal, immunocompetance is restored (de Martin et al, 1987). Immunosuppression is characteristic of lung cancers, one of the leading types of cancer in North America (Haskell et al, 1987). Moreover, systemic failure of the immune system is found in the late stages of cancer (Lee et al, 1985). If immunosuppression underlies compromised host resistence, then one might expect that attempts to overcome it may be successful. Thus, additional supportive evidence is found in the literature demonstrating promising attributes of immunotheraputic methods which employ systemic boosters such as IL-2, IFN, TNF or BCG (Hollinshead et al, 1987,

Rosenberg, 1988, Lotze et al, 1988). Due to toxic side effects of these therapies, *in vitro* methods to activate patients' lymphocytes to autologous tumors have been recently tested. Objective remissions were obtained in 14 of 41 cancer patients evaluated in a study by Muler and Rosenberg (1986). More recent work by the Rosenberg group has demonstrated the promise of immunotherapy based on the *in vitro* activation of tumor infiltrating lymphocytes, as well as showing that the efficacy of such therapy was improved by use of cyclophosphamide (discussed in Culliton, 1989). Others have shown that the effectiveness of a tumor vaccine in which irradiated patient's tumor cells are injected with BCG, was also enhanced when cyclophosphamide was given ahead of time to block the activation of suppressor cells (discussed in Cipra, 1989). Although it remains unknown why cures are still not achieved in most cases, an explanation may be that the endogenous local immunosuppression might also downregulate activated lymphocytes or that the lymphocytes are not homing to the tumor. Alternatively, it should be realized that if cell surface tumor-specific antigens do exist on all malignantly transformed cells (ie. according to the immune surveillance hypothesis, Smith et al, 1970), then cancer patients may be predisposed to autoimmune disease in addition to the postulated immunosuppression associated with the presence of the tumor. Hence, it would be conceivable that immunoenhancing therapies might exacerbate the condition of the host, rather than lead to the rejection of the tumor. Regardless of the improvements in potency or efficacy of immunostimulants, the majority of end stage cancers might not be expected to respond to this type of therapy. As well, accumulating evidence suggests that some tumors may be innately resistent to at least some immune mediators which may also account for failures. Thus, the inability of immunoenhancing therapies to effectuate cures in the majority of cancer cases cannot be taken as evidence against the immune surveillance hypothesis. Indeed overall, there is no convincing evidence contradicting the probable influence of the immune system (innate and/or adaptive) on the oncogenic outcome of tumors. On the other hand, with the exception of certain tumors, there is little evidence for the presence of tumor-specific antigens in commonly occurring human cancers. If rejection of tumors does not occur through tumor

associated antigens, then the lack of tumor-specific antigens remains the foundation for the rejection of the immune surveillance hypothesis.

C. TUMOR ANTIGENS

Tumor Specific versus Tumor Associated Antigens

In the previous section, direct and indirect evidence was given supporting the proposal that the immune system, both the adaptive and innate, may determine the outcome and growth of transformed cells. Inherent to this proposal is that the immune system can recognize neoplastic cells. Intuitively then, there must be some cell surface distinction between tumor cells and normal cells, as Ehrlich first proposed some years ago, (reviewed in Ehrlich, 1956). As a corollary, the immune system has some way to distinguish these differences.

The types of tumor markers have been broadly categorized into two main classifications; "tumor-specific" antigens (TSA's) and tumor associated antigens (TAA's). TSA's are not found or expressed in any other tissue in the host or at any stage of development. Examples of these antigens include virally encoded and mutagenically induced ones (ie. not in the genetic makeup of the host), and also the special case of idiotypes on certain T or B cell lymphomas. The adaptive immune response, by way of its rearrangement of immunoglobulin genes and T cell receptors can specifically recognize these antigens as foreign, and immunization against them leads to the rejection of such tumors. Conversely, TAA's represent abnormal expression either qualitatively or quantitatively on transformed cells. Usually these are oncofetal or differentiation antigens not normally associated with most adult tissues or alternatively with a restricted distribution in the adult. Although it is not clear, it is thought unlikely that these antigens could provoke a true adaptive immune response since the antigens are found on normal tissues and thus, are defined as self. Consistent with this, spontaneously arising tumors are less immunogenic than deliberately induced tumors, and in general, harbour almost exclusively TAA's as evidenced by the paucity of TSA's for human tumors (Nelson et al, 1987, Zalcberg et al, 1985, Virji et al, 1988, Ting

et al, 1989, McCarty et al, 1988, Klavins, 1989, Frankel et al, 1987, Borek, 1985, Hakomori, 1988, 1985, 1984, Feizi, 1985a/b, Feizi et al, 1987a/b, 1984, Reading et al, 1985, Sulitzeanu, 1985). Theoretically, an artifically induced immune response to TAA's would elicit autoimmunity. In terms of active immunotherapy, it is unclear as to whether the inducible immune response to these antigens is desirable or even possible. For example, alpha-fetoprotein, considered a prototype for tumor associated antigens, is not immunogenic in the host (Springer 1984, Coggin 1986) unless first chemically modified. Using this protocol along with adjuvants, recently a mouse anti-serum has been generated to murine AFP (Dr. Mary Crainie, personal communication). Thus in general, most probably TAA's are not immunogenic in the host of origin.

True Oncofetal Antigens: Redefined

Coggin (1986) has attempted to more precisely define tumor antigens based on their apparent immunogenicity. The complaint has been that the terms oncofetal, embryonic and differentiation antigens are used interchangeably to describe what appear to be functionally distinct antigens. Coggin suggests that oncofetal antigens (OFAs) such as the p53 and the 44 and 200 kd antigens, which are not found on any normal adult tissues, are functionally different from differentiation type tumor antigens as they elicit immune responses in the syngeneic adult host. Differentiation antigens he argues, should be further defined in terms of their expression during embryo-fetal stages such as the stage-specific embryonic antigens (SSEA) or adult-tissue restriction such as the T9-T10 human lymphocyte maturation antigens. Differentiation type tumor antigens are not autoantigenic in the native host under normal circumstances which clearly distinguishes them from true OFAs. On the other hand, fetal (FAs) or embryonic antigens (EAs) are not found in cancers but are immunogenic in the adult as they are not expressed in adult tissue. By this classification scheme, the only true oncofetal antigens are those which are autoantigenic in the adult. This places the classical 'oncofetal antigen', alpha-fetoprotein (AFP) into the differentiation category.

Under this more precise definition of tumor antigens Coggins suggests that the following are true oncofetal antigens: the 44 and 200 kd, TAFA I and II (10 kd and 4 kd), p53-55 with only the phosphorylated protein being the tumor antigen, a 120 kd glycoprotein designated 5D4 isolated from mouse embryonal carcinoma cells and the XOFA isolated from mouse fetal liver (from Coggin, 1986). Other potential true oncofetal antigens are the F9 (80 kd) antigen of teratocarcinomas, a PNA reactive 60-70 kd doublet isolated from a human melanoma cell line called M14 FA for which naturally occurring antibodies (IgG and IgM) are apparent, and possibly the TF and Tn antigens for which the carrier proteins (or molecules) have not been determined but for which naturally occurring antibodies exist (from Coggin, 1986). Springer (1984), has suggested that the TF and Tn antigens are stage-specific antigens of the human fetus disappearing prior to tolerogenic development phases. Thus, according to Coggin, few true oncofetal antigens have been discovered.

In summary, in this section the nature of tumor antigens was explored. In terms of the functional relationship to the immune system and thus to tumorigenesis, three categories of tumor antigens in general were resolved: "tumor-specific", tumor associated (or also called differentiation antigens), and true oncofetal antigens. However, before detailing the tumor antigens pertinent to this thesis: the Thomsen-Friedenreich (TF) antigen, alpha-fetoprotein (AFP) and the alpha-fetoprotein receptor/binding protein, the few tumor antigens for which a biological function has been ascribed, will briefly be discussed.

D. BIOLOGICAL SIGNIFICANCE OF TUMOR ANTIGENS

The advent of monoclonal antibodies in 1975 (Kohler and Milstein) signalled a new era for biological investigations. Despite this, and despite the number of new tumor associated antigens identified by monoclonal antibodies, comparatively very little is known about the function of tumor cell surface antigens which may mediate transformation, tumorigenesis, malignant potential or metastasis (Borek, 1985, Sulitzeanu, 1985, Sirica, 1989, Sher et al, 1988, Mc Carthy et al, 1985, Liotta, 1988, Groner et al, 1988, Greaves, 1984, Epstein, 1988, Chan et al, 1987, Burger et al, 1988, Barsky, 1988, Bishop, 1985). It has been claimed that the

majority of monoclonal antibodies made to tumor antigens are to carbohydrate antigens (Reading et al, 1985, Feizi et al, 1987a, 1987b, 1985a, 1985b, 1984, Hakomori, 1988, 1985, 1984, Coggin, 1986). However, since carbohydrates have been implicated in a plethora of important biological interactions, too numerous to list here, one can only surmise that the glycosylation changes associated with tumorigenesis might be important to the altered biological behaviour of tumor cells. At present it is not clear if this change reflects incomplete glycosylations, altered transferase levels, or if it represents the de novo expression of cell surface glycoproteins or of glycosyltransferases. Possibly, it reflects all of these.

By functional criteria, tumor antigens can be grouped into the following: oncogene products, growth factors and receptors, adhesion and metastasis associated molecules, regulators of these, and molecules associated with immunosuppression. For a more detailed, comprehensive review of the pathobiology of neoplasia, the reader is referred to an excellent text (Sirica, 1989).

Oncogenes

Studies on viral transformation led to the discovery of virally encoded transforming genes or *v-onc*'s. For the more than 20 *v-onc* genes discovered, in all cases a homologous counterpart has been demonstrated in the normal genome, (Chan et al, 1987) and these are referred to as *c-onc*'s. It has become accepted that viruses such as the retroviruses pick up these genes from normal cells. Although the proliferation and/or differentiation of normal cells involves *c-onc*'s, there is evidence for the amplification or increased levels of oncogenes or their products in cancer (Gill et al, 1987). For example, members of the *ras* gene family are the most frequent and widely distributed oncogenes in human and animal cancers (Burger et al, 1988, Bos, 1989). However, most oncogene products are restricted to the cell's interior casting doubt on the efficacy of whether or not they can serve as tumor markers *in vivo*. Cytoplasmic associated products of oncogenes such as for the *ras* gene family, *src*, *mos*, *abl*, *fes*, and *mil* all apparently induce growth factor secretion (Roberts et al, 1986). Many of these are protein kinases, usually tyrosine kinases but some such as the *mos*, *raf* and *mil* are

serine/threonine kinases. The *ras* gene family is considered an intermediate in intracellular signalling and binds GTP. Nuclear associated oncogene products (*myc, myb, fos, ski*) do not induce growth factor synthesis but sensitize cells to the action of growth factors (Roberts et al, 1986). Thus oncogenes can be broadly classified into three classes: those with protein phosphotransferase activity, those which bind GTP and those which bind DNA (Gill et al, 1987). Some oncogene products are not restricted to the cell interior. These products have been found to be growth factors (*sis* is homologous to a chain of the platelet derived growth factor) or to their receptors (*v-erbB-1* is a truncated epidermal growth factor receptor).

One might argue that oncogene products, particularly those associated with the interior of cells, are not conventional tumor associated antigens. Recently this notion has been disputed. Imaging of tumors was possible employing MAbs to the *c-myc* product (Chan et al, 1987) suggesting the potential of oncogene products as tumor markers. However, it was not excluded that imaging might have been made possible due to the expression of a secreted cross-reacting 40 kd molecule, and not because the MAb was specific for the *c-myc* product itself. Others have demonstrated inhibition of *in vivo* tumor growth by employing MAbs to the *neu* oncogene product, a 185 kd transmembrane glycoprotein tumor antigen (Drebin et al, 1986). In this case, it is likely a conventional tumor associated antigen.

Nevertheless, in some cases the overexpression of certain oncogenes correlates to certain physiological changes (Burger et al, 1988). For example, *c-myc* expression correlates to histological grading (Chan et al, 1987). *N-myc* amplification correlates to inhibition of Class I expression and tumorigenesis (Bernards et al, 1986, Brodeur, 1987, Seeger et al, 1985). The overexpression of EGFr in cancers may be associated with the downregulation of MHC Class I antigens by EGF (Pekonen et al, 1988). It is anticipated that other biological changes associated with the overexpression of some oncogene products will be forthcoming.

Growth Factors and their Receptors

The autocrine hypothesis of cancer cell growth, formulated to explain the deregulated growth of cancer cells, was originally based on the discovery of an alpha-transforming growth

factor (TGF-A, Todaro et al, 1980, DeLarco et al, 1978), and has been extended to include the concept that malignant transformation may also be the result of failure of the cells to respond to negative growth factors (Weinstein, 1988, 1987, Keski-Oja et al, 1988, Klein, 1987, Marshall, 1987, Weinberg et al, 1989), as has been postulated for TGF-B (Sporn et al, 1988, 1987, Roberts et al, 1988, Massague, 1987). Several growth factor receptors are tyrosine kinases, such as PDGF, EGF, insulin, CSF-1, (Bishop, 1985). Binding of the appropriate ligand is known to stimulate the tyrosine kinase activity of the receptor often by autophosphorylation (Deuel, 1987). Tyrosine kinase activity of oncogenes has been shown to be essential for transformation (v-*src*, *v-abl*, *v-fms*, *v-fes/fps* and polyoma virus middle T antigen (Kaplan et al, 1987)). An 85 kd phosphoprotein has been implicated as common to a number of oncogene activation pathways and is believed to be a phosphatidylinositol kinase (Kaplan et al, 1987). For the most part, growth factor receptors are not considered as tumor associated antigens but in certain cases they may be altered in association with other tumor associated antigens.

It has been recently appreciated that novel carbohydrate structures which appear on certain histological types of cancers and not found on the corresponding normal tissues may occur on growth receptors. MAbs to the tumor EGFr of the human epidermoid cancer cell line A431, have been shown to recognize blood group related carbohydrate structures (Gooi et al, 1985a, 1985b, Childs et al, 1984), such as Lewis^a and difucosylated structures of the Y type (Basu et al, 1987, Feizi et al, 1987a, 1987b) and these MAbs can activate the EGFr. The immediate significance of this is not known but might relate to alteration of the function of the receptor and at least in theory, antibodies to such carbohydrate or cross-reactive structures, might immunopotentiate tumor growth. There is evidence that EGF receptors are expressed in natural history cancers (Bauknecht et al, 1989), and the activation of the EGF receptor can be associated with the modulation of HLA Class I antigens (Hosoi et al, 1988).

Both the EGF and the PDGF receptors appear to be modulated in function by the presence of tumor associated glycolipids. The de-N-acylation of gangliosides at the cell surface can modulate growth receptor phoshorylation. GM3 was able to inhibit the EGFr

without interfering with EGF binding (Hanai et al, 1987, Bremer et al, 1986).

Other potential growth factors and/or receptors which might be tumor associated antigens but for which this question has not been addressed include proteins of molecular weights of 60, 52, and 30 kd, secreted from breast cancers, the last one which competes for EGF receptors (Dickson et al, 1987). Antibodies to a 68 kd cell-surface molecule on breast cancers not found on normal lymphocytes inhibited tumor cell growth and adhesion (Kaniyama et al, 1987).

MAbs to the transferrin receptor although not a true growth factor receptor can inhibit growth possibly by interference with iron transport and can be used to image tumors or for immunotherapy (Trowbridge, 1983).

There does exist a family of tyrosine kinase receptors which have received much attention in the past few years as they appear to relate to oncogenesis. This family is the *c-erbB* oncogene family of which *c-erbB-1* is the Epidermal Growth Factor Receptor, and *neu* is *c-erbB-2*. The importance of these kinases particularily to breast cancers was first suggested by the finding of gene amplifications (Slamon et al, 1987) and which has been confirmed in many laboratories. The overexpression and/or gene amplification of *c-erbB-2* has been demonstrated to have prognostic significance (Slamon et al, 1987, 1989, Tandon et al, 1989, Wright et al, 1989, Berger et al, 1988, Patterson et al, submitted). This oncogene appears to be expressed in 90 % of breast cancers (King et al, 1989). On the other hand, *c-erbB-1* is expressed in about 33 % of breast cancers, shows a non-linear negative correlation to ER/PgR status, but may also show some prognostic significance (Pekonen et al, 1988, Bäuknecht et al, 1989, Grimaux et al, 1989).

Using molecular genetics approaches, the malignancy-conferring property of *c-erbB-2* has been clearly demonstrated (Di Fiore et al, 1987, Lee et al, 1989, Lehvaslaiho et al, 1989). There have even been reports that this oncogene may be causally related to breast cancer as a single step induction of mammary carcinoma was found in transgenics (Muller et al, 1988); although others have contested this point (Bouchard et al, 1989). Given that this is the first time that a non-stochastic induction of cancer has been claimed for a single oncogene such as

evidenced through the use of transgenics under a relevant promotor such as MMTV, it serves to underscore the importance of determining what the ligand is for the orphaned tyrosine kinase, *c-erbB-2*. Whether there may be antigenic changes to this tyrosine kinase receptor upon malignant transformation, remains to be determined.

Adhesion and Metastasis

The notion that molecules which play a role in adhesion or cell motility, may play an important role in invasion and metastasis and thus contribute to malignant transformation, has become a central tenet of oncogenesis (Sher et al, 1988, Barsky et al, 1988, Van den Hoff et al, 1988, Varani et al, 1989, McClay et al, 1987). Receptors for extracellular matrix proteins such as fibronectin and laminin, have recently received much attention in this regard (Liotta, 1988, Liotta et al, 1986, Martin et al, 1987, McCarthy et al, 1985). Of the several laminin receptors, some of which are members of the integrin family of cell adhesion proteins (Gehlsen et al, 1988), the high affinity laminin receptor, first isolated in 1983 (Rao et al), has been implicated in migration (Wewer et al, 1987, 1986) and in chemotaxis and haptotaxis (Liotta, 1988). A pentapeptide has been identified from the laminin B1 chain which specifically binds to the high affinity 67 kd laminin receptor (Graf et al, 1987a, 1987b), and which also mediates its biological effects. Not surprisingly though, it is the number of unoccupied laminin receptors which correlates to metastasis (Hand et al, 1985) and a receptor distinct from the high affinity laminin receptor appears to effect motility on laminin (Castronovo et al, 1989b). Very recently the 67 kd laminin receptor has been sequenced (Yow et al, 1988). This receptor is unusual since it apparently lacks a transmembrane segment and a typical signal sequence. It also lacks N-glycosylation sites, and contains a single disulphide bridge (Yow et al, 1988). This laminin receptor is expressed on a vast majority of carcinomas and here it shows an uniform membrane distribution. In contrast, on normal breast tissues it associates with the basement membrane (Hand et al, 1985). It is also expressed on motile cells of the immune system such as macrophages.

Receptors for certain matrix proteins might be tumor associated antigens. Cheresh and co-workers have found that MAbs to GD2, a tumor associated antigen of small cell lung cancer and neural crest tumors, affects the binding capacity of these receptors (Cheresh et al, 1986). Hyaluronate binding proteins which may also bind to fibronectin, laminin and collagen (Turley et al, 1984), stimulates cell motility (Turley, 1982, Turley et al, 1985), possibly through a protein kinase (Turley, 1989) or through association with *K-ras* (Turley et al, 1989). This antigen may be expressed in melanomas and in sarcomas and is thought to contribute to metastasis.

Mammalian lectins particularly those with affinity for galactose residues have been implicated in metastasis. Cells exhibiting higher levels of galactose-specific lectins were found on cells with higher lung-colonizing potential (Raz et al, 1986). These have also been implicated in growth control and cell adhesion (Allen HJ et al, 1987, Carding et al, 1985, Lotan et al, 1985).

On the other hand, loss of *Ulex europeus I* binding in human breast cancers has been correlated to metastasis (Walker et al, 1986). Higher serum levels of tissue polypeptide antigen, a differentiation antigen, correlates to metastasis (Khanna et al, 1987).

Carcinoembryonic antigen (CEA) is a well characterized 180 kd tumor associated antigen, expressed in most colon cancers, and belongs to a family of intercellular recognition molecules which includes the immunoglobulin supergene family of which N-CAM is also a member. Recently, its has been shown that CEA mediates homotypic binding of human colon adenocarcinoma cells and rodent cells transfected with CEA cDNA (Benchimol et al, 1989). It has been postulated that CEA, via homotypic intercellular adhesion may promote metastasis since cell aggregates which may break away from the primary tumor may have a greater chance of survival in the circulation and/or through lodging in other organs. It has also been suggested that the inappropriate expression of CEA may disrupt the normal architecture of colonic epithelium and may allow for more cellular movement which again might contribute to invasion or metastasis (Benchimol et al, 1989).

PNA reactive glycoproteins have also be implicated in adhesion and metastasis (Irimura et al, 1984) and will be considered under the section on the TF antigen.

Immunosuppressive Factors

One of the most intriguing and perhaps paradoxical findings to emerge concerning tumor associated immunosuppressive factors is that some of these appear to be able to not only downregulate the immune system but also the growth of the cancer cells from which they are supposedly derived. Transforming Growth Factor-Beta (TGF-B) is one such factor. It is ubiquitously expressed, and high affinity receptors have been found on all normal and malignant tissues tested (Roberts and Sporn, 1986, Sporn et al, 1988, 1987, Massague, 1987), with notable exceptions of retinoblastomas (Kimchi et al, 1988), or hormone-dependent breast cancer cell lines (Arteaga et al, 1988). TGF-B is thought to participate in a negative autocrine or feedback inhibition of cell growth, however in cancer it is thought a lesion occurs which permits the uncontrolled growth of transformed cells (Roberts et al, 1988, Knudson, 1989, Levine et al, 1989). Different lesions have been found in cancers (Huang et al, 1988, Knabbe et al, 1987, Kimchi et al, 1988) including loss of receptors or the production of a TGF-B binding protein which renders it latent (Wakefield et al, 1988, Miyazono et al, 1988). Thus, while TGF-B is not considered a tumor associated antigen, the lesion in the negative autocrine function may be considered an important facet of malignant transformation of epithelial cells.

The immunosuppressive nature of TGF-B has only recently been appreciated and it is also apparent that immunocytes synthesize and secrete TGF-B (Kehrl et al, 1986, Assoian et al, 1987). Receptors for TGF-B are upregulated after T cell activation and TGF-B inhibits the IL-2 induced upregulation of the IL-2 and the transferrin receptor (Kehrl et al, 1986). T cells upon activation produce mRNA for TGF-B but its secretion is not detectable until after 2 to 4 days in culture (Kehrl et al, 1986). TGF-B secretion appears to be in a latent form (Sporn et al, 1987). Alpha-IFN augmented natural killer activity has been also shown to be inhibited by TGF-B but not that augmented by IL-2 (Rook et al, 1986, Katz et al, 1988).

Effects on lymphokine-activated killer cells has also been shown (Espevik et al, 1988). The proliferative response of B cells is similarly inhibited by TGF-B (Petit-Koskas et al, 1988, Kehrl et al, 1986). The effects of TGF-B on macrophages are multifactorial. TGF-B is the most potent chemotactic substance known (Wahl et al, 1987). At higher concentrations, TGF-B induces mRNA for IL-1 (Wahl et al, 1987) but paradoxically blocks the ability of IL-1 to stimulate lymphocyte proliferation (Wahl et al, 1988). Expression of mRNA for TGF-B is constitutive whereas secretion of an active moiety appears to be related to the activation of monocytes (Assoian et al, 1987). TGF-B inhibits the CSF-1 dependent proliferation of macrophage bone marrow precursors and a CSF-1 dependent macrophage cell line (Strassmann et al, 1988) and deactivates macrophages (Tsunawaki et al, 1988). TGF-B also inhibits tumor necrosis factor (TNF) production by macrophages and the expression of Class II antigens in melanomas and adherent cells derived from PBMC (Czarniecki et al, 1988). Overall, TGF-B seems to inhibit both cellular and humoral activity.

Although the immunosuppressive attributes of AFP will be discussed in detail later, another molecule also produced by breast cancer cells has been associated with immunosuppressive activity. Pregnancy associated alpha-2 glycoprotein serum levels are increased not only in pregnancy but also in breast cancer patients. Serum levels can correlate to the clinical course of breast cancers and the preclinical recognition of their metastases (reviewed in Sarcione et al, 1983b). It has been postulated that this protein plays a role to prevent the immunorejection of breast cancers (Sarcione et al, 1983b).

Other immunosuppressive factors associated with human cancers include a 50 - 70 kd fraction of adult T cell leukemic (ATL) cells (Shirakawa et al, 1986). Sometimes a tumor associated antigen is thought to induce the release of nonspecific suppressor factors from normal human PBL's as has been suggested for CEA which is about 50 kd (Medoff et al, 1986). There are reports of other immunosuppressive moieties made by cancer cells but whether they are tumor associated needs to be examined. In most cases they are not well characterized.

Summary

For the most part an understanding of the biological significance of tumor associated antigens has eluded tumor biologists. In some cases a role in transformation has been established but not a role in oncogenesis. Despite the prevalent finding that MAbs to tumor associated antigens tend to be to carbohydrate structures, qualitative differences to normal tissues have not been described in many if not most cases, a notable exception being the EGF receptor. Most regretably, an ubiquitously expressed immunosuppressive factor has not been demonstrated to be produced by malignant tumor cells and thus may question the validity of the immune surveillance hypothesis.

E. THE THOMSEN-FRIEDENREICH ANTIGEN

The Tumor Specific TF Antigen Defined By Naturally Occurring Antibodies

The Thomsen-Friedenreich phenomenon was described over half a century ago (Friedenreich, 1930) referring to the polyagglutinability of red blood cells upon storage. It was then discovered that contaminating microbes produced neuraminidase which removed sialic acid from glycophorin and thus, unmasked the normally cryptic TF antigen. Since there are anti-TF antibodies in all normal adult sera, these red cells were agglutinated by all sera tested.

The TF hapten has been shown to involve gal-beta(1-3)-alpha-galNAc O-linked to glycophorin by inhibition assays performed with isolated naturally occuring antibodies (NAbs), in neuraminidase treated erythrocyte assays (Uhlenbruck et al, 1969, Longenecker et al, 1984, Bray et al, 1981, Hoppner et al, 1985, Wolf et al, 1986, 1987), or in neuraminidase treated lymphocyte assays (Bray et al, 1981, Longenecker et al, 1984). However, in other assays on neuraminidase treated lymphocytes, the specificity of the NAbs were found in one case to include both Gal-1-3-galNAc and Gal-beta(1-4)-gluNAc NAbs (Rogentine, 1974). In assays performed on purified desialyated glycophorin, Springer et al (1975), reported that the best inhibitors of the NAbs involved gal-beta(1-4)-GluNAc although at 500 times the

concentration, gal-beta(1-3)-GalNAc also did inhibit. Therefore, although it is generally accepted that the TF hapten involves Gal-beta(1-3)-GalNAc, evidence has also indicated that it may also involve NAbs to Gal-beta(1-4)-GluNAc which is the single N-glycan also found on glycophorin unmasked by neuraminidase treatment (Dahr et al, 1975, and see discussion in Uhlenbruck et al, 1969 and in Prokop et al, 1969).

Naturally occurring antibodies have been also demonstrated to the precursor of the TF antigen, the Tn antigen, which is comprised of alpha-galNAc O-linked to serine/threonine. The use of these NAbs by absorption techniques, as for the TF antigen, have shown the incidence of expression of the Tn and TF antigens in approximately 90% of common, human adenocarcinomas (Springer, 1974, 1976, 1979, 1984, 1985a, 1985b, 1985c, 1986, 1989).

Several authors have now attempted to identify in more detail, the specificity of naturally occurring anti-TF antibodies (Hoppner et al, 1985, Wolf et al, 1987). By the classical definition, these are antibodies which react with neuraminidase treated human red blood cells but not with untreated RBC's, and appear to involve the specificity, galactose(beta)-1-3-galNAc. The immunodominant determinant of the TF antigen involves not only the TF carbohydrate hapten, but the conjugation mode to the protein appears to be also important (Hoppner et al, 1985). There appears to be a heterogeneous population of antibodies in normal human serum. Wolf et al, (1987), attempted to separate these into 3 groups with distinct specificity patterns, none of which were inhibited by a naturally occurring glycolipid, monosialo-GM1. The ability of naturally occurring antibodies (NAbs) to bind to the TF antigen of tumor cells seemed to coincide with the inhibition of binding with nitrophenyl-beta-galactoside. This confirms the previous work of Longenecker et al (1984, Rahman et al, 1982) which showed that a MAb, 49H.24 which had clear specificity for the alpha TF anomer, and was not as well inhibited by nitro-phenyl-B-galactose, did not react with tumors. On the other hand, MAb 49H.8 which had some sensitivity to inhibition by both anomers of the TF hapten but was more sensitive to inhibition by the nitro-phenyl-B-galactose or phenyl-B-galactose carbohydrates, was able to recognize tumor cell lines or neuraminidase treated lymphocytes. However, others (Howard et al, 1980a) found

that IgM anti-TF corresponding to the TF-1 antibodies of Wolf et al, which were found not to be inhibited by nitro-phenyl-B-galactoside, could discriminate malignant from non-malignant breast tissues as tested by immunohistological means on paraffin sections. These results suggest the presence of at least two types of anti-tumor-TF antibodies which can be discriminated by their inhibition with phenyl-B-galactosides.

The appearance of the TF antigen on human cancers was first reported by Springer et al, (1974, 1975, 1976) for breast cancer tissue. Employing the Peanut Agglutinin (PNA) lectin, which binds to the TF antigen, several investigators have confirmed that the vast majority of human adenocarcinomas and some leukemias express the TF antigen (Howard et al, 1980b, Noujaim et al, 1983, Ghazizadeh et al, 1985, 1984, Orntoft et al, 1984, 1985, Lloyd et al, 1984, Matalanis et al, 1986, Yuan et al, 1986, Cooper et al, 1987, 1983). In an interesting study on the cell surface changes of normal colon cells transformed by SV40, Moyer et al, (1984) demonstrated that the appearance of PNA reactive determinants was an early event in the transition to the malignant phenotype.

Springer et al, have found that approximately 90% of common human adenocarcinomas express the TF/Tn antigens but deduced that these antigens are not expressed on normal tissues (1974, 1975, 1976, 1984, 1985a, 1985b, 1985c, 1986, 1989). The methodology used here was an absorption of titred naturally occurring antibody onto the test tissue, and therefore might be an underestimation of the expression of these antigens particularly for normal tissues, when compared to more direct techniques. Not surprisingly, other workers using either the PNA lectin or monospecific rabbit antisera to the TF antigen have found normal tissues to express the TF antigen, albeit in a limited or restricted distribution. For example, in normal breast or benign breast tissue, cytoplasmic or luminal staining of ductules can be demonstrated, whereas in cancer cells, surface staining is evident using the PNA lectin (Howard et al, 1980b). However, since the lectin is not exclusive to the TF antigen and similarly, it is possible that the rabbit antisera might crossreact with other galactose bearing determinants, the use of monoclonal antibodies (MAbs) or the use of labeled naturally occurring antibodies of particular mono-specificity, should clarify this point.

Attempts to Characterize TF Antigens by the Use of MAbs

Our laboratory has made MAbs to TF or TF-like antigens using several approaches. The first approach taken was to generate MAbs to the cryptic TF determinant on red cells. Both the 49H.24 and 49H.8 IgM MAbs were made to neuraminidase treated human red blood cells (Rahman et al, 1982, Longenecker et al, 1984). While the 49H.24 MAb appeared to involve specificity for the alpha TF hapten and did not react with tumors, the 49H.8 MAb reacts with tumors and neuraminidase treated lymphocytes. It appears to involve a beta anomer TF-like antigen since its binding is completely abolished by phenyl-beta-galactose (Longenecker et al, 1984). Studies with the 49H.8 MAb showed that this determinant in fixed sections was expressed in 76% of colon tumors and showed reactivity with 81% of pre-malignant polyps and no reactivity with normal colon. In contrast, PNA reactivity was noted in 91% of colon tumors and 68% in normal mucosa (Yuan et al, 1986). A polyclonal antibody to the cryptic TF antigen of red cells showed intermediate staining (Yuan et al, 1986). In another study involving human cell lines, PNA reactivity demonstrated for the 9 cell lines tested, was enhanced by neuraminidase treatment, whereas 5/9 cell lines had 49H.8 reactivity which did not show enhancement by neuraminidase treatment. In this study, a rabbit antiserum or human anti-T did not react with cell lines, with or without neuraminidase treatment (Wolf et al, 1986). Furthermore, as a difference in sensitivity to the effects of trypsin and pronase treatment on the binding of the PNA lectin versus the 49H.8 MAb was demonstrated, these results may suggest the non-identity of PNA receptors to the 49H.8 reactive determinant. Again, on fixed tissues, Wolf et al (1988) investigated the expression of the 49H.8 determinant. With the exception of positive staining in normal glomeruli and tubuli of the kidney, no staining of normal adult tissues was demonstrated. While all breast cancers examined seemed to have at least weak expression of the 49H.8 reactive epitope, strong staining of the primary tumor seemed to positively correlate to tumor progression. Although 26-34% of breast cancers without lymph node involvement stained with the 49H.8 MAb, 42% of the primary tumors with lymph node involvement were reactive with the 49H.8 MAb, as were 50% of the metastasis. The apical/luminal staining typified by the 49H.8 MAb would be

consistent with it being a secreted molecule. In a separate study on colon carcinomas, while no staining of normal colon was noted, the 49H.8 MAb detected 71% of the cancers (Itzkowitz et al, 1989). To summarize, these results suggest that the 49H.8 MAb shows immunoreactivity in 26-76% of common human adenocarcinomas, may be a secreted glycoprotein as shown by its sensitivity to pronase (Wolf et al, 1986, 1988), may not be identical with PNA receptors (Wolf et al, 1986, Yuan et al, 1986), and its expression may correlate to risk of pre-malignant colonic conditions (Yuan et al, 1986), or may correlate to breast cancer tumor progression (Wolf et al, 1988). Furthermore, the expression of this antigen, although rather specific to tumors might be found in some normal adult tissues such as in kidney (Wolf et al, 1988).

The tumor antigen reactive with the 49H.8 MAb might be a 800-1000 kd mucin (Samuels et al, 1990) which would be consistent with the likehood that it is a secreted glycoprotein.

A second approach has been to generate MAbs to synthetic TF and Tn antigens. Others have also synthesized these for similar purposes (Diakun et al, 1987). The Tn antigen as already mentioned, is the precursor to the TF and may also be expressed in about 90% of common, human adenocarcinomas (Springer et al, 1989, 1986, 1985a, 1985b, 1984). The relative overexpression of the Tn to the TF antigen in breast cancers often shows a correlation to anaplasia and to a poorer prognosis (Leathem et al, 1987, Springer et al, 1989, 1986, 1985a, 1985b, 1984), when demonstrated by the *Helix pomatia* lectin or when inferred by the absorption of the NAbs. On the other hand, *Vicia villosa* lectin which may detect both the alpha and beta anomers, appears to detect normal colon in addition to its reactivity with 50-80% of colon tumors (Itzkowitz et al, 1989).

155H.7 is an IgG MAb made against the synthetic TF antigen conjugated via a ceramide type linkage. It strongly reacts with about 89% of carcinomas but also maintains reactivity with normal tissues (Longenecker et al, 1987, 1988). The antigen detected by this MAb has not been characterized. Similarily, 164H.1 made to the alpha anomer of the Tn antigen (glycoprotein type linkage), reacts with about 80% of adenocarcinomas but shows

considerable heterogeneity. For example, in breast cancers 73% showed positive reactivity but 85% of these showed heterogeneity. Again, this MAb shows some specificity for normal tissues and the tumor associated antigen has not been characterized (Longenecker et al, 1987).

Others have generated MAbs to the naturally occurring tumor TF and Tn antigens derived from *in vitro* propagated cell lines. MAb RS1-114, raised against a human lung adenocarcinoma cell line seems to be similar to the 49H.8 MAb, as it reacts with cryptic TF determinants on red cells and lymphocytes, reacts with tumor cell lines and carcinomas and is inhibitable by phenyl-B-galactose (Stein et al, 1989). In contrast to the 49H.8 MAb, its reactivity is enhanced by neuraminidase treatment and detects 74% of carcinomas but about 85% of breast cancers. It reacted with normal breast and kidney, however. This antigen has not yet been characterized.

Takahashi et al (1988), have produced a MAb, CU-1 to a high molecular weight antigen secreted by a lung squamous cell carcinoma which appears to have affinity for an alpha Tn antigen. The CU-1 MAb reacts with approximately 88% of breast and colon cancers but also reacts with 33% of normal breast or colon tissues and unlike other MAbs to the Tn antigen (Hirohashi et al, 1985, Clausen et al, 1988), does not cross-react with blood group A.

In a subsequent article, two monoclonal antibodies with reactivity to sialosyl-Tn were reacted against various colon type tissues. Here, these MAbs did not react with normal adult colon and reacted with 100% of well or moderately differentiated colon carcinomas, and 75% of poorly differentiated colon tumors (Itzkowitz et al, 1989). One of these, B72.3 detects a high molecular weight mucin (Nuti et al, 1982, Schlom et al, 1987).

To summarize, it seems that the use of hybridoma technology has so far failed to identify "tumor-specific" TF or Tn antigens, present in 90% of adenocarcinomas, as Springer has proposed might exist. Many of the MAbs presently available seem to react with high molecular weight secreted mucins but which can be present in normal tissues. It is interesting that serum complexes containing the TF/Tn antigens and NAbs have not been demonstrated (Dube, 1987). This raises the possibility that the TF and Tn antigens are not "tumor-specific" (Barr et al, 1989).

The Sneaking Through Hypothesis for the TF/Tn Antigens of Springer

In the previous section, it was discussed that the TF/Tn-like antigens have been characterized as mucins. The presence of these extracellular or shed antigens may abrogate the effectiveness of a humoral or cellular response and so it is argued, may permit the tumor to "sneak past" the immune system (Springer, 1984). However, the presence of the DTH reaction in presumably all the cancers which express the TF/Tn antigens argues against the notion that the tumor has sneaked past the immune system. As mentioned previously, it seems that the TF/Tn antigens have not been isolated as a serum complex (Dube, 1987), suggesting that they are not likely secreted or shed. Thus, shed TF/Tn antigens are not likely the reason for the ability of the TF/Tn antigens to foil the immune response. As well, the DTH reactivity might be taken as evidence that the TF/Tn antigens are most likely cell surface expressed. It is more likely that a local immunosuppressive environment surrounds the tumor, and this may relate indirectly to the presence of the TF/Tn antigens or to malignant transformation. Until the TF/Tn antigen is characterized, the point of how its expression may relate to anergy of the host immune system will remain unaddressed.

The Potential Role of the TF/Tn Antigens in Invasion or Metastasis

Terminal galactose residues are not normally accessible either on glycoproteins or on the surface of cells. Liver contains various lectins with specificity for galactose residues (Ashwell et al, 1982). There is increasing evidence for other lectins with specificity for other monosaccharides such as fucose and in the liver uptake of L-fucose-alpha1-3-GluNAc proteins ie. lactoferrin (Prieels et al, 1978). Kupffer cells as well as hepatocytes bind neuraminidase treated red blood cells or lymphocytes (Kolb et al, 1979). The existence of hepatic lectins for terminal galactose residues might implicate these in mediating liver specific metastasis. In this regard, the ability of the RBC TF antigen to inhibit the binding of highly metastatic lymphoma cells to hepatocytes in a dose responsive manner has been demonstrated (Springer et al, 1983). Importantly, low metastatic, parental lymphoma cells do not adhere to hepatocytes. However, when the parental lymphoma cell line was compared to the metastatic

variant, it was not clear whether the metastatic variant contained significantly more TF/Tn structures or not, as demonstrated by the absorbtion of the NAbs (Springer et al, 1983). In fact, this point was mentioned in a later article (Springer et al, 1985a). Thus, this might be taken as evidence that the tumor TF/Tn antigen on these lymphoma cells does not react with the NAbs purified on desialyated glycophorin or that these lymphoma cells do not express the TF/Tn antigen. Indeed, the omission of data supporting the ability of the NAbs to inhibit hepatocyte:tumor cell adherence may reflect this point. However, since desialyated glycophorin inhibited this response, it may rather be that terminal galactose partakes in hepatocyte adherence and may not necessarily relate to the presence of the TF/Tn antigen on lymphoma cells.

The citation that the TF/Tn antigens may play a role in invasion and metastasis as evidenced by the above experiments is not only inexact but would be an oversimplification. Invasion and metastasis is a multi-complex cascade with requirements for detachment from the extracellular matrix and for directed (chemotaxic, haptotaxic) or non-directed (chemokinesis) movement. The carcinoma must be able to also cross several basement membranes and endothelial cell layers. By the time the carcinoma is in the liver parenchyma and growing, the specific interaction of carcinoma cells with hepatocytes may be inconsequential or may have played a minimal role in extravasation of the tumor.

In rodent models where the role of the TF/Tn bearing antigens in metastasis can be most easily studied, the expression of a 580 kd TF/Tn bearing mucin of a mammary adenocarcinoma has been correlated to liver metastasis (Steck et al, 1987). Apparently a similar 550 kd antigen is expressed in human breast cancers (Dr. G. Nicolson, personal communication). The expression of the approximately 500 kd TF/Tn antigen shed by TA3-Ha cells (murine mammary adenocarcinoma) known as epiglycanin (epi) is associated with tumor aggressiveness and liver metastasis (Van den Eijden et al, 1979). The 49H.8 MAb produced in this laboratory to the cryptic red cell TF antigen has been shown to specifically inhibit the binding of RAW-117 Ha cells (a murine, liver metastasing variant of a T cell lymphoma) to liver endothelial cells (Dr. Ed Roos, personal communication). The latter might suggest the

presence of a lectin on endothelial cells which might interact with high molecular weight mucins. These rodent models provide more convincing evidence that these high molecular weight mucins might play an important role particularly, in liver specific metastasis. Although the interaction may merely require terminal galactose residues, and thus may not exclusively implicate the TF or Tn antigens per se, the fact may remain that these antigens can play a role in liver specific metastasis.

Potential Significance of Heightened Tn Expression to Tumor Progression

Again by absorption of the NAbs, Springer et al have shown an increased relative expression of Tn to TF antigens in human breast cancers in association with more invasive cancers (ductal versus lobular) and for murine TA3-Ha (more aggressive) to the TA3-St parental line (Springer et al, 1985a). Despite the claim that this study showed more Tn than TF in 92% of primary anaplastic breast cancers (Springer, 1989), this point was not made clear by the data given. Instead the 6 metastatic lesions studied showed equivalent levels of both the Tn and TF antigens (Springer et al, 1985a).

However, by an independent group employing the *Helix pomatia* lectin (HPA), which has specificity for terminal alpha galNAc and thus probably recognizes the Tn antigen, a positive correlation of HPA reactivity and aggressiveness of breast cancers was established (Leathem et al, 1987). This applied to both disease-free intervals and to survival. In a separate report on urinary bladder transitional cell carcinomas, 81% of patients with *Vicia villosa* agglutinin (VVA) positive tumors suffered from invasive recurrence. In a subgroup of the patients with no TF antigen demonstrable by PNA reactivity but which were cryptic TF antigen positive (ie. PNA reactive after neuraminidase treatment), 5/6 of these for which VVA reactivity was also demonstrated, showed invasive recurrence (Nishiyama et al, 1987). The follow-up period was a minimum of two years. There was also a correlation of VVA reactivity to grade of the tumor. Grade I was associated almost exclusively with cryptic TF antigens and no VVA reactivity. Grade 2 was associated preferentially with cryptic TF antigens (60%) but also 34% had expression of the VVA determinant. In Grade 3, 71% of the

tumors had VVA reactivity. It is important to note that in this study all tumors with the expression of VVA determinants also expressed cryptic TF antigens, as revealed by PNA reactivity upon neuraminidase treatment.

In analogy to TF antigens, the Tn antigen may similarly be substituted with sialic acid ie. alpha(2-6). Two MAbs, TKH2 and B72.3 are thought to detect sialosyl-Tn (Itzkowitz et al, 1989). Interesting results with these MAbs were obtained when compared to the 49H.8, the CU-1 MAbs and the VVA lectin. First of all, the TF antigen as demonstrated by the use of the 49H.8 MAb, and sialosyl Tn antigens were not detected in 22 cases of normal colon whereas Tn reactivity as detected by the VVA lectin was found in 14/22 cases. It is not clear if this latter observation would represent a cross-reaction to blood group A antigens. The expression of the TF antigen seemed to show a distinct positive correlation to differentiation and this was less evident for the expression of the sialosyl-Tn. The expression of the Tn antigen varied from 50 to 80% of the cancers tested, and the VVA lectin showed 100 % reactivity with the fetal colonic specimens tested (Itzkowitz et al, 1989).

The B72.3 MAb originally described by Nuti et al (1982) has been subsequently shown to have specificity for sialosyl-Tn. It reacts with a 200-400 kd protein complex and is expressed in 46% of breast carcinomas and 62% of metastatic lesions. It was shown to react with 2/15 benign breast lesions but not with normal tissues.

Taken together, these studies with colon tumors, breast cancers and particularly with urinary bladder cancers, support the contention that the less mature glycoform, the Tn antigen, may correlate to more invasive tumors, metastasis, shorter disease free intervals, shortened survival times, invasive recurrence, and/or less differentiated tumors. Sialosyl-Tn or the expression of the 49H.8 reactive determinant, may in contrast, be associated with more differentiated tumors. Nevertheless, the reports of the correlation of 49H.8 reactivity with the progression of breast cancers and particularly to metastasis (Wolf et al, 1988), suggests that the TF antigens may play some role in metastasis which has been supported by rodent models.

Paradoxical Nature of Incomplete Glycosylations For the Unmasking of TF and Tn Antigens in Cancer

It has been said the most common alteration in cancers (Greaves, 1984) or of surface carbohydrates (Reading et al, 1985) concerns the appearance of underglycosylated carbohydrate structures on lipids and/or proteins. An early observation concerning a block in glycosylation pathways in rat mammary carcinomas (Keenan et al, 1973) had suggested that certain glycosyltransferases might be depressed in cancer cells and this may lead to the unmasking of certain carbohydrate antigens. However, this observation pertained to glycolipids and not to glycoproteins. Subsequently, it has been repeatedly shown that in the serum of cancer patients fucosyl, galactosyl and sialyl transferases are elevated as well as in the carcinoma cells (reviewed in Dube, 1987 and Alhadeff, 1989). Thus, it seems paradoxical, that there seems to be unmasking of the TF and Tn antigens in malignantly transformed cells but yet these cells have increased transferase activity. Indeed, cancers which show the presence of the less mature isoforms eg. Tn, also have TF or cryptic TF structures (Springer 1984, 1985, Nishiyama et al, 1987) suggesting that the relevant enzymes are present and active. Furthermore, not all glycoproteins undergo incomplete glycosylations and often glycosylated variants containing added residues are detected as the aberration (reviewed in Alhadeff, 1989). Thus, the reduced activity of the relevant transferases cannot explain the incomplete glycosylations associated with the expression of the TF and Tn antigens in malignancy.

Another widely held view was that malignant cells proliferate at such a high rate that there is not the time to properly complete the glycosylations, such as envisaged for the TF/Tn antigens (reviewed in Reading et al, 1985). However, again this explanation cannot account for the specificity towards the TF/Tn antigen as one might expect a plethora of antigens to be similarly affected. Furthermore, it does not readily explain the relationship of heightened unmasking of the Tn to the TF antigen with tumor progression which does not necessarily, directly reflect proliferation rates. Other postulates although more nebulous, may paradoxically be more plausible. These (Noujaim et al, 1983, Greaves, 1984) ascribe differences in cell surface carbohydrates to reflect the differentiation status of the cell, which

may be more "fetal-like". These postulates are more in keeping with the current beliefs that the malignant phenotype results from specific aberrations in the DNA with the associated deregulation of cellular senescence and/or failure to differentiate rather than resulting from a primary problem with uncontrolled growth stimulation such as mutations in growth factor receptors, etc..

At present, one mechanism which might account for the unmasking of the TF and Tn antigens in malignant cells but not normal cells, may be the *de novo* expression of high molecular weight mucins specific to carcinomas. However, these mucins have been demonstrated to be also expressed in normal tissues. On the other hand, these mucins cannot account for the unmasking of the TF and Tn antigens in 90% of common, human adenocarcinomas since they are not expressed in 90% of common, human adenocarcinomas, and it has not been conclusively resolved if these are the TF/Tn antigens or merely cross-reactive ones.

Although there is yet no direct evidence for this, the specific changes leading to the expression or unmasking of the TF/Tn antigen could result from the binding activity of some protein which becomes co-expressed in malignantly transformed cells and which blocks the glycosylation of the TF/Tn hapten-bearing protein because it specifically binds only this protein. Although not quite analogous, there is a similar precedent set for the case of the glycosylation status of an IgE binding protein, which in turn either enhances or suppresses IgE synthesis (reviewed in Rademacher et al, 1988). This hypothesis is attractive since it may not only explain the specific unmasking of the TF/Tn antigens as might only occur in malignant tissues but the increased expression of this "TF/Tn antigen specific binding protein" might lead to the relative heightened expression of the Tn antigen with tumor progression. The association of the expression of the postulated binding protein with tumor progression might directly or indirectly relate to a role of this protein in conferring tumor aggressiveness. However, the elucidation of how the TF and Tn antigens become unmasked in carcinomas awaits the identification of the TF/Tn antigen and the characterization of the postulated binding protein, if indeed one exists. Since none of the anti-TF NAbs were inhibited by a

glycolipid, monosialo-GM1 (Wolf et al, 1986), this may suggest that the TF antigen is most likely a glycoprotein and as such, may be subject to "incomplete glycosylations" through steric hindrance mechanisms operative during post-translational modifications.

Summary of Investigations with Antibodies

To conclude this section on the TF and Tn antigens, it seems some investigators have advocated that a "tumor-specific" TF/Tn antigen appears to become cell surface expressed in 90% of common, human adenocarcinomas. There seem to be IgM NAbs in the sera of all normal adults to this or cross-reactive antigens. This apparently "tumor-specific" TF/Tn antigen, probably a glycoprotein, has not been yet identified or characterized. Several high molecular weight mucins bearing TF or Tn like structures have been identified by the use of MAbs, but most often seem to be associated with more differentiated tumors and are not expressed in 90% of adenocarcinomas. However, these mucins seem to play a role in metastasis, particularly liver. Until the putative "tumor-specific" TF/Tn antigen expressed in 90% of common, human adenocarcinomas is identified and characterized, the mechanisms leading to "incomplete glycosylations" will remain elusive. On the other hand, an elucidation of a mechanism which can explain malignancy-specific "incomplete glycosylations" of a PNA-reactive glycoprotein, as it might occur in 90% of adenocarcinomas, may lead to the identification of Springer's elusive TF/Tn antigen.

PNA Reactive Glycoproteins in Malignancy

In order to address the significance of the expression of the TF antigen in malignancy, these antigens must be identified and biologically characterized. There has not yet been the unequivocal demonstration of a tumor antigen which bears the single disaccharide, gal(beta)1-3-galNAc O-linked to serine/threonine, for example as demonstrated by chemical analysis. As previously mentioned, although the PNA lectin reacts preferentially with this or the alpha anomer disaccharide, it can also include other terminal galactose bearing glycans. Thus, although the use of the PNA lectin has its limitations, since the TF antigen is PNA

reactive, the use of this lectin could provide the initial identification of candidate TF antigens.

In this regard, by immunohistology employing the PNA lectin, PNA receptors appear to be expressed 90% of common, human adenocarcinomas (Bocker et al, 1984, Holt et al, 1984, Howard et al, 1980b, Kellokumpu et al, 1987, Newman et al, 1979, Orntoft, 1984, Orntoft et al, 1983, Rhodes et al, 1986, Shysh et al, 1985, Fozard et al, 1987); in other cancers (Ariano et al, 1985, Kagawa et al, 1985, Levin et al, 1980, Newman et al, 1983, Reisner et al, 1979, Studer et al, 1987); or found cryptic on red cells (Springer et al, 1982, Uhlenbruck et al, 1969); as well as benign tissues (Howard et al, 1980b); and some normal adult tissues (Limas et al, 1986, London et al, 1978, Picard et al, 1983, 1979, Rosenberg et al, 1985), which include human peripheral adherent cells, but not T or B cells. There are several high molecular weight human tumor associated antigens which have been shown to be PNA reactive: ie. the 320 kd Ca-1 antigen (Wiseman et al, 1984, Ashall et al, 1982, McGee et al, 1982), a 550 kd moiety (Dr. G. Nicolson, personal communication) and the DF3 antigen, a 330-450 kd mucin (Sekine et al, 1985, Kufe et al, 1984). It has not been addressed whether these antigens are widely expressed in most histological types of cancer, nor if there are naturally occurring antibodies to these antigens.

Other indirect studies suggest a role of PNA-reactive tumor associated antigens in tumorigenesis. For example, studies on growth potentials of human malignant melanoma cell lines selected for their subcutaneous growth in nude mice indicated that a 190 kd PNA reactive band was only detected in the low growth capacity group while a 60 kd antigen bearing cryptic PNA reactive sites was expressed to higher levels in the high growth capacity group (Berthier-Vergnes et al, 1986).

In brain tumors, but not in normal grey or white matter, PNA reactive high molecular weight antigens were found by western blotting at 260 kd and a major band at 180 kd. A 70 kd moiety was also found only in tumor extracts (Davidsson et al, 1987). Others have found a series of small molecular weight antigens (26, 32, 33, and 50 kd) associated with colonic tumors (Kellokumpu et al, 1987).

Recently, a 68 kd surface-labeled molecule has been immunoprecipitated from human leukemic cells using the PNA lectin and a rabbit antiserum was made (Studer et al, 1987). While both human thymocytes and leukemic cells (ALL) expressed a 150 kd PNA immunoprecipitated antigen, the antiserum to the 68 kd molecule reacted only with leukemic cells. Most interesting, autophosphorylation analysis indicates that the 68 kd, but not the 150 kd antigen, has protein kinase activity suggesting a possible role in oncogene activation. It would be interesting to determine if this rabbit antiserum reacts with 90% of common human adenocarcinomas or if this 68 kd molecule is related to the 60 kd one associated with high growth capacity of melanomas as described by Berthier-Vergnes and co-workers (1986). There are also similarities amongst these PNA reactive antigens and the PNA reactive doublet of Gupta et al, (1983a, 1983b) which migrates between 60 and 70 kd for which there are naturally occurring antibodies of the IgM and IgG isotypes.

In summary then, although PNA reactivity is associated with 90% of common human adenocarcinomas, and some PNA-reactive tumor associated antigens have been described, none of these has yet been shown to be expressed in 90% of common, human adenocarcinomas. With the exception of the PNA-reactive doublet of Gupta et al (1983a/b), naturally occurring antibodies recognizing PNA-reactive tumor antigens have not yet been identified. It remains to be determined whether a single, PNA-reactive, tumor associated antigen expressed in 90% of common adenocarcinomas exists for which there are naturally occurring antibodies.

In an effort to address this question, monoclonal antibodies (MAbs) were generated to PNA reactive proteins derived from breast cancer membranes (biopsy materials). During the course of this investigation, a novel, cell surface expressed, PNA-reactive, tumor associated antigen was discovered by its reactivity with the 167H.1 and 167H.4 MAbs and was found to be expressed in 90% of common, human adenocarcinomas. Various lines of evidence will be presented which suggests that suggest that this 67 kd receptor may be the elusive, alpha-fetoprotein receptor/binding protein which appears to be PNA-reactive. Thus, this necessitates a review on alpha-fetoprotein and its receptor/binding protein in order to place

these findings in their proper perspective.

F. ALPHA-FETOPROTEIN

Alpha-fetoprotein (AFP) was the first tumor associated antigen described (Abelev, 1963, Tatarinov et al, 1964) shortly following its discovery in 1956 by Bergstrand and Czar as a major alpha migrating protein of fetal serum.

AFP concentrations, at the mg/ml level, peak in fetal serum at 12 to 14 weeks gestation, and may comprise up to 10% of the total fetal proteins (Ruoslahti et al, 1971). Thereafter the concentrations decrease to trace adult levels (less than 30 ng/ml) at about 5 weeks after birth. After the first year of life, AFP is diagnostic for primary cancer of the liver, malignant embryonal teratomas (Ruoslahti et al, 1971), yolk sac tumor of the ovary, gastric and pancreatic tumors (Mizejewski, 1985), and in disorders in which proteases spill into the circulation such as pancreatic insufficiency, cystic fibrosis (Chandra et al, 1975), and liver cirrhosis, (Mizejewski, 1985).

Human AFP is a sialyated single chain glycoprotein with an apparent molecular weight of 67,500 daltons under reducing conditions. Under nonreducing conditions it migrates at about 55 kd. The odd migration pattern of AFP in SDS-PAGE is thought to be due to the unusually high number of disulphide bonds. This pattern is similar to that of serum albumin which is evolutionarily related (Tamaoki et al, 1983; Morinaga et al, 1983, Ruoslahti et al, 1976). AFP contains approximately 580 amino acids and contains 3.6% carbohydrate by weight which are N-linked (Yachnin et al, 1977, Ruoslahti et al, 1979, 1978). Human AFP is not PNA reactive (Ruoslahti et al, 1979, Breborowicz, 1988). The isoelectric points of human AFP are 4.85 (major) and 5.2 (minor), (Smith et al, 1980), and the former variant can be shown to contain fatty acids (Parmelee et al, 1978). This former variant, the most electronegative form, contains the more potent immunosuppressive subpopulation (Lester et al, 1978, 1977). Total desialyation of AFP alters but does not abolish the heterogeneity seen by isoelectric focussing. In these cases, the AFP species focus at a higher pH (Smith et al, 1980). In humans, the pH 4.85 fraction has been shown to be twice as immunosuppressive as

the the pH 5.2 and unlike murine AFP, complete desialyation did not affect the immunosuppressive activity of human AFP (Smith et al, 1980, Lester et al, 1978). AFP tends to form aggregates and the oligomer formation increases with freeze/thaw cycles (Smith et al, 1980). The sedimentation constant for human AFP is 4.5 to 5.0 Svedburg units with a stokes radius of 3.26 nm and the extinction coefficient is 5.3 to 5.26 at 278 nm (Mizejewski, 1985).

Although AFP and human serum albumin (HSA) are homologous by several criteria, antisera made to AFP or HSA do not cross-react unless the antisera is made to the denatured molecules (Ruoslahti, 1979). AFP's of all species, except human, have a high affinity for estrogen (Uriel et al, 1976), whereas albumins from different species do not exhibit such affinity. This difference is believed to be due to changes in the first domain (amino terminus) where the greatest differences lie (Baker, 1987). In addition, AFP shows an affinity preference for polyunsaturated fatty acids, mainly docosahexenoic and arachidonic acids (Jacobson et al, 1983a, Uriel et al, 1987), and may thus play a role in the delivery of fatty acids during embryogensis when biosynthetic pathways are still inefficient (Naval et al, 1987). AFP differs from HSA in that the latter is not glycosylated (Kerckaert et al, 1977). The serum half-life of AFP is 3.5 to 5.5 days whereas it is 15 to 19 days for albumin, perhaps reflecting a receptor mediated uptake in the former case.

Both AFP and HSA have been sequenced (Morinaga et al, 1983) and their relateness established. The AFP and HSA genes have been localized to the q11-24 portion of Chromosome 4 in humans, and are also found linked in the mouse on Chromosome 5 (Minghetti et al, 1983, Harper et al, 1983). Recently, the entire sequence of the AFP gene has been sequenced (Gibbs et al, 1987), as well as the 5' flanking region (Sakai et al, 1985) and enhancer sequences (Watanabe et al, 1987). AFP, unlike albumin, is a glucocorticoid suppressed gene as measured by reduced mRNA levels after after dexamethasone administration to newborn rats (Cote et al, 1984, Wang et al, 1987, Guertin et al, 1983). However, *in vitro* studies on rat hepatoma cell lines indicates that dexamethasone treatment can both enhance (McA-RH8994) or inhibit (Morris hepatoma 7777) the levels of mRNA for AFP (Chiu et al, 1986). A protein has been postulated to be involved in the regulation of the

AFP gene since cycloheximide, which blocks protein synthesis, and excess progesterone block the ability of glucocorticoids to alter AFP gene expression. Indeed, recently a 149 kd nuclear protein which specifically binds cloned AFP DNA has been isolated from AFP synthesizing HCC cell lines, not by non-synthesizers or by normal adult liver (Wang et al, 1987).

Three major functions have been associated with AFP (Mizejewski, 1985, Tilghmann, 1985): i) the carrier-transport of various ligands, ii) the regulation of the immune response and iii) the regulation of cell growth in normal and neoplastic tissue.

Rodent AFPs bind estrogens while human AFP does not demonstrate measurable affinity (Ruoslahti et al, 1979, Nunez et al, 1987, 1974), although there has been some suggestion that a subpopulation of human AFP may bind estrogen. Albumin and AFP bind bilirubin, copper, as well as fatty acids, although for AFP these are mainly unsaturated. Human AFP binds dyes such as bromosulfophthalein, rat AFP binds aflatoxin B, and rodent and human AFP bind prostaglandins (Smith et al, 1980). Bovine, rodent and human albumins bind Cibacron Blue, whereas AFP from these species do not, and therefore provides a convenient method for their separation. AFP subpopulations are thought to bind and thus inactivate proteases (tryptases), and can be eluted from trypsin affinity columns by either high salt or by benzamidine, a specific trypsin inhibitor (Mizejewski, 1985). In addition to 0.4 M KCl, the protease inhibitors also convert the 8S cytosolic AFP-complex to the immunoreactive 4S (Mizejewski, 1985). Baker (1987) has recently compared the sequences of HSA and AFP to human tissue-type plasminogen activator (a serine protease) and found some regions of homology suggesting these sequences may be evolutionarily related. Interestingly, part of this segment on tissue-type plasminogen activator is related to epidermal growth factor (Baker, 1987). It is difficult to presently explain that AFP which may have some sequence homology to serine proteases can at the same time, have binding properties thought to suggest that it is a serine protease inhibitor.

The concept that AFP might be immunosuppressive dates back to the 70's when Murgita and Tomasi (1975a/b) showed that murine AFP depressed the plaque forming response to sheep red blood cells and various mitogenic responses, such as Con-A, PHA, LPS

and also for MLRs. Later in 1976, Murgita and Wigzell showed that the antibody response to DNP-Ficoll (a T-independent antibody response) and the polyclonal antibody response to LPS was not inhibited, which suggested that B cells are not directly affected by AFP. Further, the work of Keller and Tomasi (1976) showed that AFP could be demonstrated on splenic lymphocytes undergoing graft-versus-host reactions by immunofluorescence. By SDS-PAGE analysis of ^{14}C labeled cells and immunoprecipitation it was inferred that AFP was produced by activated lymphocytes. However, since that time numerous reports have indicated that studies of the effects of AFP on proliferation of non-homologous sources of lymphocytes such as those employed for mitogen reponses or MLRs may be misleading, as it is thought AFP stimulates the proliferation of suppressor cell populations (Alpert et al, 1978) and inhibits autoreactive and/or Ia restricted reactions (Mizejewski, 1985, Hooper et al, 1987). Thus, various studies have implied that AFP can suppress and/or enhance mitogenic responses (Yachnin, 1976, Yachnin et al, 1976), but most often its effects are found to be dose dependent (Mizejewski, 1985, Ruoslahti et al, 1979, Charpentier et al, 1977). To also contribute to the complexity of the situation, Ruoslahti et al (1979) have pointed out that these assays were conducted in the presence of FCS which contains over 1 mg/ml of bovine AFP. In addition, whether or not differences in sialation, fatty acid and/or prostaglandin content could explain the aberrant results have not been resolved. For example, Deutsch (1983) provided convincing evidence that the suppression of the human PHA response by AFP was not significant even at levels of 1 mg/ml unless the AFP was first refatted with arachidonic acid. Suppression under these conditions thus went from 15% to 75% of the total response. This author proposed that the conversion of arachidonic acid to prostaglandins in macrophages might account for this suppression. However, as Yachnin (1983) points out, the refatting of AFP does not always renew its suppressive activity. Alternatively, the isolation procedures differ amongst groups and it is unknown if other molecules contaminate these various preparations. For instance it is conceivable that a suppressive factor might have the same apparent molecular weight as AFP or alternatively might have such potent suppressive activity that protein stains of SDS-PAGE gels may not detect the low levels present.

Charpentier et al (1977) have suggested that AFP preparations might be contaminated by a truly suppressive molecule. Thus, it has been found that the immunosuppressive effects of AFP often disappear upon purification (Ruoslahti et al, 1979). The ensuing batch variations may be explained by elution with denaturating agents (Calvo et al, 1985) which may to a various extent irreversibly destroy the biological activity of AFP or dissociates or destroys the proposed contaminating molecule. Further details on other possible explanations for these discrepancies are discussed by Soubiran et al (1979).

Nevertheless, in keeping with the notion that tolerance either to self or to maternal antigens must be established, Murgita and co-workers have provided much evidence that AFP might play a dominant role to this effect. For example, the proliferation of autoreactive Lyt 1,2 thymic T cells are the most sensitive to AFP inhibition while alloreactive thymic neonatal reactions are less so, and adult splenic alloreactions were found to be the least (Hooper et al, 1987). Of interest, while this was true for thymocytes, Lyt 1 splenocytes from neonates were concomitantly resistent to the effects of AFP as measured by ^{3}H-thymidine incorporation. Further, they indicate that the antiproliferative effect on thymocytes also occurs in the presence of added IL-2 as well as blocks the elaboration of IL-2, but yet does not compete for IL-2 receptors. Thus, they conclude AFP inhibits a critical stage of the activation sequence of Lyt 1 rodent, thymocytes (Hooper et al, 1987). Previously, they showed that AFP acts on a monocyte-enriched MLR-stimulating cell population which in turn activates the T suppressor cell circuit of splenocytes (Peck et al, 1982). Overall then, these authors imply that rodent thymic T cells are inhibited in the presence of AFP and for mature lymphocytic responses, AFP may affect the activation sequence through the indirect activation of T suppressor cells via its inhibitory effects on monocytes. Lu and coworkers (1984) shed some light on this subject when they showed by immunofluorescence and radioimmunoassay that AFP (crude or purified) prevented lymphokine induced Ia expression of murine peritoneal macrophages measured on day 5, which was not affected by indomethacin. Indomethacin prevents the generation of prostaglandins thus this eliminated the possibility that prostaglandin E2 might have mediated these effects. More recently, it has been shown that AFP can downregulate the

constitutive expression of Class II antigens on macrophages and post-induction levels, but unlike other antagonists, AFP fails to inhibit gamma-IFN mediated induction of Ia antigens (Crainic et al, 1989). Lu and coworkers have suggested that the delayed ontogenesis of Ia-bearing macrophages; governed in part by elevated AFP levels and in part by prostaglandins, might contribute to the development of self-tolerance. More recently, the inhibitory effects of AFP on macrophage activation has been extended to include tumoricidal activity (Lu, 1986).

Although NK cells are present in the neonate, there is a significant lack of NK activity during this period. NK cells lyse some but not all tumor targets as well as bone marrow and thymic cells of murine or human origin. This suggests that an oncofetal antigen may serve as a target antigen. NK cells have been implicated as playing a role in the regulation of hematopoietic stem cells. Soluble agents which prevent the expression of NK mediated cytotoxicity include estradiol, hydrocortisone, B glucan, and prostaglandins (Cohen et al, 1986). It was recently shown by Cohen et al (1986) that AFP can inhibit the IL-2, poly I:C and interferon mediated activation of NK cells in a time and dose related fashion (Hooper et al, 1987). This finding again underscores the possibility that AFP may play an important role in the generation of self-tolerance and attests to its potential immunosuppressive qualities.

Further adding to the notion of AFP playing a role in tolerance, it has been suggested that the administration of AFP may suppress some autoimmune diseases (Mizejewski, 1985, Abramsky et al, 1982, Brenner et al, 1984, 1980, Buschamn et al, 1987). AFP was found to suppress both the early acute and late chronic phases of experimental autoimmune myasthenia gravis (Brenner et al, 1984). However, it remains unclear why select sources of AFP should inhibit the binding of antibodies to the acetylcholine receptor *in vitro* while AFP from the serum of hepatoma patients, isolated by acid elution of an immunoaffinity column would not (Brenner et al, 1984). Since patients with certain autoimmune diseases such as myasthenia gravis and rheumatoid arthritis (Mizejewski, 1985) undergo temporary remission during pregnancy, the possibility of a therapeutic effect of AFP deserves further attention.

The third major function of AFP involves growth regulation. As pointed out previously, AFP may allow the preferential activation and proliferation of T suppressor cells through the downregulation of Ia antigens on antigen presenting cells. It might also directly inhibit the proliferation of rodent thymic Lyt 1 cells. Recently, it has been shown that two populations of bone marrow cells, distinguished by their Soybean Agglutinin lectin reactivity, are stimulated to proliferate in response to AFP. They appear to have differential kinetics suggesting they are discrete populations. Both may be defining natural suppressor cells (Hoskin et al, 1985, Hamel et al, 1987).

Outside the immune system, AFP has been shown to indirectly regulate growth of normal and neoplastic tissues. AFP is capable of suppressing estrogen-induced growth of the immature mouse uterus (Mizejewski, 1985, Mizejewski et al, 1983), the *in vitro* growth of cultured estrogen sensitive tumor cells (Mizejewski, 1985, Mizejewski et al, 1987), and the *in vivo* growth of rat mammary tumors (Jacobson et al, 1984, 1983b). It is believed that this mechanism does not involve estrogen sequestration since human AFP which is thought to not bind estrogen (Nunez et al, 1974), mediates these affects and agonists of estrogen which do not bind to AFP also function (Sheehan et al, 1987).

To coincide with the inconsistent results discussed previously for the immune system, Mizejewski et al, (1987) found that the condition or the purity of AFP is also significant for its effects on estrogen mediated responses. Freshly isolated AFP is anti-proliferative for immature uterine growth while old, denatured or aggregated AFP might potentiate growth with exogenous estrogens. The reasons for these discrepancies have yet to be elucidated but may relate to oligomer formation of the AFP receptor by cross-linked AFP. This might occur in much the same way monoclonal antibodies can behave as agonists or antagonists to cellular growth receptors, a function which is often dependent on the ability to cross-link receptors.

AFP was thought to be the only steroid-binding plasma protein not responsive to its ligand; i.e. estrogen, in rodents (Sheenan, 1987). However, large doses of estradiols induce serum AFP levels in adults both in males and females (Hau et al, 1984). Others, (Kotani et al, 1984) have shown that a single intraperitoneal injection of estriol (E3) increased the serum

AFP concentration and hepatocyte proliferation both of which peaked on day 5 in the adult mouse. The association of increased levels of AFP with proliferation has also been reported for human hepatomas. Bromodeoxyurindine preferentially labeled transplanted human hepatoma cells which contained cytoplasmic AFP as analysed in frozen sections (Sasaki et al, 1987). Taken together these results imply that there is a close association of the presence of AFP, probably involving endogenous production, with the growth of hepatocytes whether or not they appear to exhibit estrogen sensitivity. However, the finding that exogenous AFP might downregulate the growth of estrogen sensitive tissues needs to be reconciled with the above findings concerning the endogenous production and positive growth regulation. Other estrogen sensitive tissues have also been shown to produce AFP such as the MCF-7 human breast cancer cell line (Sarcione et al, 1987b, 1985). Recently this group has proposed that AFP synthesis by breast cancer cells might be involved in the stimulation of breast cancer proliferation but the endocytosis of exogenously added AFP would give a downregulatory signal for estrogen mediated growth. However, no underlying mechanism was suggested (1987b, 1985). This group has also provided evidence that serum levels of AFP are elevated above normal for breast cancer patients (Sarcione et al, 1987a). The reason that this had never been demonstrated before is because the analysis of serum levels of AFP have always measured free AFP levels and not total which can be revealed by 0.4 M KCl.

It is not yet clear whether AFP directly stimulates the autocrine growth of cancer cells. In hepatoma models, the expression of AFP seems to correspond to active proliferation, but most hepatomas do not seem to express the AFP receptor (Ruoslahti et al, 1978, Hosokawa et al, 1989), ruling out an autocrine growth stimulation in these cases. However, human hepatocellular carcinoma (HCC) is unusual to most cancers as it involves a viral etiology involving hepatitis B and non-A, non-B viral infections (Zhang et al, 1990). Furthermore, unlike other solid tumors, loss of heterozygosity at the region encoding AFP is found in 40-50 % of informative cases (constitutional heterozygotes) of virally associated HCC (Zhang et al, 1990), potentially suggesting the AFP gene might be specifically altered in the development or progression of HCC but not other human cancers. How this may relate to

proliferation of hepatoma cells needs to be resolved.

It would be very important to discern whether or not AFP (or mutationally activated AFP receptors) can contribute to the "uncontrolled growth" of cancer cells through autocrine mechanisms. However, by far the majority of evidence suggests that exogenously added AFP may inhibit growth, at least for estrogen sensitive tissues (Soto et al, 1980, Sonnenschein et al, 1979, 1980). Thus, the claim that AFP might be a growth factor for bone marrow derived natural suppressor cells (Hoskin et al, 1985, Hamel et al, 1987), deserves further attention. As will be presented later in the thesis, it appears for the most part that AFP and AFP agonists down-regulate proliferation and/or differentiation signals indicating that AFP is not likely a bona fide growth factor. How a factor might have negative growth regulatory properties yet in more unusual circumstances appear to enhance the growth of certain cells, and how a factor might be associated with enhanced *in vivo* growth without mediating a direct growth signal are points which will be covered later in the thesis. For the present moment, it is sufficient to note that despite the evidence that AFP expression appears to correspond to an increased growth potential of cancer cells, it may not impart a growth signal directly.

In order to resolve whether AFP in an autocrine loop contributes to the malignancy of the tumor it will be necessary to assess the levels of mRNA for AFP as well as test for the presence of the AFP-BP/AFPr by immunological techniques. Immunological techniques fail to assess the expression of AFP in cancer tissues because its presence appears to be masked by the soluble AFP-BP and the presence of cytoplasmic AFP does not distinguish endogenous production from that which is specifically taken up by receptors. Only *in situ* hybridization studies for mRNA for AFP on sections will clarify this point. Sarcione et al have previously shown the presence of complexed AFP in all breast cancer cytosols tested (1983a, Biddle et al, 1987). It would be of additional interest to determine if AFP plays any role in the switch of breast cancer cells to hormone independence, or if it can account for the reduced risk of breast cancers in those women who have had children before the age of thirty, as proposed by Mizejewski (1985).

It is important to note that increased AFP levels can precede the clinical appearance of hepatoma even by 18 months (Chen et al, 1984), and increased serum levels of AFP often are associated with a poorer prognosis (Mahour, 1988, Malogolowkin et al, 1989). Indeed, particularly for liver metastasis, distant metastases late in tumor progression have been shown to be associated with grossly elevated levels of serum AFP in most cancer types (Morimoto et al, 1988). Thus, a correlation of AFP expression to malignant transformation and/or upregulation with tumor progression may occur for most solid tumors. *In situ* hybridizations for mRNA for AFP and the semi-quantitation of its levels may confirm this point.

Other tissues apparently sensitive to estrogens include the hematopoietic tissues. Hayama et al (1983) have shown that treatment of mice with a large dose of E3 an analogue of estradiol, causes the appearance of hemopoietic foci in adult liver. Since NK cells regulate hematopoiesis and are known to be negatively regulated by estrogens as well as by AFP, this raises the question as to whether the colony stimulating activity of estrogens is direct or indirect. It would be interesting to determine whether bone marrow cells or cells in peripheral lymphoid organs can also produce AFP and/or are estrogen sensitive. Keller and Tomasi have already indicated that activated splenocytes might produce AFP (1976). Since AFP shares some homology with a portion of the p62 *c-myc* gene product (Chan et al, 1987) or indirectly to EGF (Baker, 1987), these results might imply that AFP might be a growth regulator. That EGF is also located on Chromosome 4 (4q25) relatively close to AFP (4q11-13) may or may not be coincidental. The direct versus indirect effects of AFP on regulation of certain genes clearly needs further study.

In summary, in the 70's the clinical relevance of the expression of AFP became appreciated and assays were developed for the post-surgical monitoring of AFP levels in cancer patients, and for the prenatal diagnosis of neural tube defects. During this same period, the AFP molecule was biochemically characterized for several species, and insight to the physiological role of AFP was obtained; first as a carrier-transport mechanism for various ligands and then as an immunosuppressive agent. By the mid 80's, AFP was suggested to have a third biological role, the regulatory control of cell growth and gene expression in normal and

neoplastic tissues. However, these effects would not be possible without a cell surface alpha-fetoprotein receptor.

G. THE ALPHA-FETOPROTEIN RECEPTOR/BINDING PROTEIN

Spearheaded by Moro et al (1981) and Uriel et al (1981), the existence of a specific receptor for AFP to explain the selective uptake of AFP by fetal cells was proposed, and AFP uptake was confirmed for developing brain (Villacampa et al, 1984a, Uriel et al, 1983a, Moro, 1983), for breast cancers (Villacampa et al, 1984b), for neuroblastoma cells (Hajeri-Germond et al, 1985), and for a rhabdomyosarcoma (Uriel et al, 1983b).

However, as early as 1976, aberrant migration patterns of AFP in electrophoretograms were found in cancer serums as well as in normals (Norgaard-Pedersen et al, 1976, Marrink, 1978). In hindsight, the gamma migrating source of AFP, most likely an AFP-protein complex, may have been the first demonstration of a soluble binding protein for AFP. Similarily in 1976, Uriel et al isolated AFP complexes in immature rat uterine cytosols, followed by Smalley and Sarcione in 1980. Again, this suggested the presence of a soluble binding protein for AFP. Functional evidence for AFP uptake and thus, the presence of AFP receptors in normal estrogen-sensitive tissues was provided by Pool et al (1978), wherein they showed the functional castration in female rats transplanted with the Morris hepatoma 7777 was due to AFP. Shortly thereafter, the isolation of AFP containing complexes was extended to cancer tissues, in particular, breast cancer (Sarcione et al, 1983a). That same year, it was proposed (Uriel et al, 1983a), that the cell surface alpha-fetoprotein receptor (AFPr) would be regulated according to the degree of differentiation of immature tissues. 1983 was also the first year that specific uptake of AFP *in vitro* by cancer cells was provided (Uriel et al, 1983b). However, the original demonstration of AFP binding to cell surfaces was by Tomasi's group (Dattwyler et al, 1975) on murine splenocytes and lymph nodes by using FITC conjugated AFP and additionally on T cell lymphomas (Keller et al, 1975, Laborda et al, 1987). In 1976, Keller and Tomasi showed the *in vivo* induction of the putative AFPr (and/or the expansion of cells expressing the AFPr) during the course of a graft-versus-host reaction

as the number of spleen cells able to bind AFP went from 22 % to 43 %. Thus the first evidence for the existence of a soluble and/or cell surface AFP-BP was provided for normal and neoplastic adult tissues over 15 years ago.

From 1983 through 1985, the radioimaging of various murine tumors by radiolabeled AFP clearly supported the existence of the AFPr: for spontaneous mammary cancers (Uriel et al, 1984), and neuroblastoma (Hajeri-Germond et al, 1985). Uriel's group not only showed that AFP accumulated around the nuclear membranes of malignant cells but that the uptake of AFP was via clathrin-coated pits, the latter strongly suggesting a receptor mediated mechanism. The ability to image tumors in this manner also suggested a) that the cell surface expression of the AFPr on normal tissues is minimal and b) the AFPr is very likely a tumor associated antigen. Breast cancer metastases were similarly imaged using ^{131}AFP in a single patient (Moro, personal communication).

Scatchard plot analysis, for the MCF-7 human mammary adenocarcinoma (Villacampa et al, 1984b) suggested the presence of both a high affinity AFPr (K_d=4.5 x 10^{-9} M, n=2000/cell) and a lower affinity site (K_d=1.3 x 10^{-8} M, n=135,000/cell). Similar results were obtained by Naval et al (1985) on a murine T cell lymphoma, YAC cells. Here however the K_d's obtained were 1.5 x 10^{-9} and 2.2 x 10^{-7} M with n = 2,000 and 320,000 respectively. However, in contrast to the earlier reports of Tomasi's group (Dattwyler et al, 1975, and Keller et al, 1976), normal adult murine T cells or thymocytes were not found to specifically bind AFP, although some binding of AFP was found on PHA activated spleen cells enriched for T cells (Naval et al, 1985, Torres et al, 1989). This discrepancy regarding the expression of the AFP receptor on developing or mature T cells warrants further attention.

In addition to the presence of a cell surface receptor with two binding affinity sites, there have also been reports of soluble AFP binding proteins found in normal serum (Sarcione et al, 1987a) and in breast cancer cytosols (Biddle et al, 1987, Sarcione et al, 1987b, 1985, 1983a). The presence of a soluble AFP binding protein was evidenced from immunological assays to detect AFP before and after 0.4 M KCl treatment. Apparently, most antibodies to

AFP do not react with AFP if it is complexed to the soluble AFP binding protein (Sarcione et al, 1987a/b, 1985, 1983a, Biddle et al, 1987) but see Hosokawa et al (1989) for a recent exception. That AFP more often than not, exists in a non-immunoreactive complex has been extensively reviewed by Mizejewski (1985).

Studies on the affinity of the breast cancer cytosolic binding protein indicated a K_d intermediate between the high and low affinity, cell surface AFP receptors ie. at 4.5×10^{-8} and the numbers of receptors per MCF-7 cell at about 75,000 (Biddle et al, 1987). Thus, as the authors state, it is not clear whether or not the breast cancer cytosolic AFP-BP is the same as the cell surface one. If it is, then this might suggest that the AFP-BP requires an AFP-BP Anchoring/Transducing Molecule (AATM) and/or that the AFP-BP may lack a transmembrane segment. The characterization of the AFP-BP and its sequence would be instrumental to resolving this point.

Another contentious issue is whether or not macrophages express a functional AFP-BP. AFP binding studies on thioglycollate stimulated peritoneal macrophages apparently gave negative results by the Tomasi group (Dattwyler et al, 1975). In view of the direct effects of AFP on the downregulation of Class II, MHC molecules on macrophages (Lu et al, 1984, Crainie et al, 1989), it is likely that at least partially activated murine macrophages express functional AFP receptors. However, due to the intrinsic problems with the purification of AFP and whether contaminating molecules promote the biological activities ascribed to AFP, the expression of AFP receptors awaits immunohistological investigations with anti-AFPr MAbs.

Although evidence implicating the existence of the cell surface AFPr and soluble AFP-BP have been accumulating since 1975, these entities have not been conclusively identified nor characterized. Gel filtration analysis has demonstrated a 130 kd complex containing AFP in rat uterine cytosols (Villicampa MJ, personal communication). This might imply that the AFP-BP may have a molecular weight of about 65 kd. However, the possibility that this represents AFP aggregation or indirect association to the estrogen receptor which is approximately 68 kd (Greene et al, 1986) has not been ruled out. An antibody specific to the

cell surface receptor which is not reactive with AFP would clarify this point. However, it has not yet been available.

Monoclonal antibodies to the AFP-BP would not only facilitate its biochemical characterization but would also be useful to discern the potential significance of the expression of the AFP-BP on malignant and non-malignant tissues. If MAbs can be shown to be AFP agonists or antagonists, they might serve to help elucidate the biological roles of AFP and the corresponding mechanisms. In addition, MAbs might be clinically useful for cancer serum screening assays or perhaps as biological response modifiers such as in the immunotherapy of cancer, autoimmunity or transplantation, depending upon whether the MAb or corresponding active peptides were AFP agonists or antagonists.

II. HYPOTHESIS AND RESEARCH OBJECTIVES

Overview, Perspectives and Theorectical Framework

Oncogenesis is a complex, multifaceted process in which little is understood about the factors which contribute to tumor progression and to the demise of the host. As an approach to elucidate those factors which govern malignant potential, the identification and characterization of a widespread, prognostic cancer marker was sought. Accumulating evidence had suggested that a Peanut Agglutinin (PNA) reactive or *gal-galNAc-O-ser/thr* bearing glycoprotein (the Thomsen-Friedenreich (TF) antigen), might be widely expressed in human adenocarcinomas, and may undergo incomplete glycosylations with tumor progression. This was postulated to result in the unmasking of terminal *galNAc-O-ser/thr* (known as the Tn antigen), which has been correlated with poorer prognosis (see literature survey). How carbohydrate unmasking may relate to malignant potential has remained an enigma. Until now, a PNA-reactive or TF or Tn bearing glycoprotein which is **expressed in 90% of common, human adenocarcinomas** has not been characterized.

In order to overcome this deficiency, we sought to identify and characterize a PNA-reactive tumor associated antigen by generating and isolating monoclonal antibodies

(MAbs) to PNA-reactive determinants from pooled, human breast cancer biopsy membranes. Two of the resulting MAbs, 167H.1 and 167H.4, were found to react with 90% of common, human adenocarcinomas by immunoperoxidase staining on frozen sections, and both were found to identify a novel, 67 kilodalton, tumor associated antigen. The PNA reactivity of the 67 kilodalton antigen was confirmed by carbohydrate inhibition testing on antigen purified to apparent homogeneity. These MAbs were interestingly also found to identify isoforms of soluble and cell surface associated alpha-fetoprotein binding proteins (AFP-BP). This was assessed by their ability to block the binding of alpha-fetoprotein (AFP) to cell surfaces, by the ability of AFP to specifically block their binding to soluble sources of the AFP-BP, by the purification of the AFP-BP on the basis that it specifically binds AFP, and on the basis that these MAbs appear to be AFP agonists. Alpha-fetoprotein, the first oncofetal antigen discovered, has been ascribed growth regulatory/differentiation properties and immunosuppressive effects, and thus is likely to be important in tumor initiation and progression in the intact host.

The use of the 167H.1 and 167H.4 MAbs has confirmed the immunosuppressive, growth and/or differentiation regulatory properties of AFP. As expected, the cell surface associated AFP-BP was detected on monocyte populations but generally not on most peripheral T cells regardless of their activation status. In contrast, there appears to be differential expression of the 167H.1 and 167H.4 reactive AFP-BP isoforms on discrete subpopulations of developing human thymocytes. The 167H.4 MAb may demark thymocytes committed to the generative pathway. These results, along with the evidence that these MAbs appear to detect glycosylation differences, suggest that the glycosylation status of the AFP-BP is differentially regulated during development.

Based on circumstantial evidence, it is proposed that the 167H.1 MAb may detect a more mature glycoform of AFP-BP than the 167H.4 MAb, and that the co-expression of AFP may through complex formation, sterically hinder the glycosylation of the soluble AFP-BP resulting in the appearance of the less mature, 167H.4 reactive glycoform. Hence, it is hypothesized that the relative unmasking of the carbohydrates on the PNA-reactive AFP-BP

may correlate to tumor progression, in that it might signify the increased expression and production of AFP relative to the amounts of AFP-BP produced. This may be functionally related to tumor progression in that the overexpression of AFP may have important autocrine activities related to the intrinsic malignancy of the tumor such as the abrogation of cellular senescence, as well as immunosuppressive paracrine properties which may eliminate host defence mechanisms and contribute indirectly to the uncontrolled growth and metastasis of the tumor. The steric hindrance mechanism of incomplete glycosylations may be the first hypothesis which attempts to relate the unmasking phenomenon functionally to tumor progression. It may also be the first explanation of how incomplete glycosylations may occur on certain glycoproteins and how this unmasking can occur in cells where there may be an increased activity or expression of the relevant glycosyltransferases.

Although not generally appreciated, there is accumulating evidence in the literature which suggests that in most cancer cell types, grossly elevated levels of AFP can be detected late in tumor progression (as reviewed in Morimoto et al, 1988), but are rarely detected prior to metastasis except in certain types of cancer such as hepatomas or germ line tumors. It is proposed that the inability to antigenically detect AFP prior to the late stages of cancer may relate to the co-expression of the soluble AFP-BP which may mask the presence of AFP until excess free AFP is produced, associated with tumor progression. Others have provided direct evidence that the AFP binding protein commonly masks the presence of AFP in human breast cancers (Biddle et al, 1987, Sarcione et al, 1987b, 1985, 1983a) or in breast cancer sera (Sarcione et al, 1987a). The above observations taken together with the findings presented in this thesis provide circumstantial evidence that the expression and function of AFP and AFP receptors may be clinically relevant to malignancy and that this property may be more universal than previously envisaged. However, direct evidence for the expression of AFP in human cancers must be addressed by molecular methods to detect mRNA for AFP, before any generalizations can be made.

Overall, the cumulative work presented in this thesis supports the notion that a widespread, PNA reactive glycoprotein plays an important role in tumor initiation and

progression of human adenocarcinomas and may provide a basis for the development of novel cancer therapies and diagnostic or prognostic markers.

The identification and characterization of ubiquitous tumor progression factors may provide valuable insight to human oncogenesis such that novel treatment modalities and/or new diagnostic or prognostic markers may be developed. We have chosen to attempt to identify and characterize a common, PNA-reactive or TF/Tn bearing, tumor associated antigen, for the following reasons. The available evidence had suggested that this antigen or its "unmasked" glycoforms may be ubiquitously expressed in adenocarcinomas, may be diagnostic or prognostic, and might pertain to metastasis or immunosuppression of the host. Therefore, PNA-reactive tumor associated antigens may directly contribute to the malignant potential of the tumor. We were also curious as to the nature of the unmasking of the TF/Tn antigen and how this might correlate to tumor progression, since this remained a major enigma. As a prerequisite to uncovering the potential role of this antigen to oncogenesis, the following hypothesis was formulated.

Working Hypothesis

A specific, PNA reactive and/or TF or Tn bearing tumor associated glycoprotein exists on the cell surface of 90 % of common human adenocarcinomas and is co-specified by the demonstration of naturally occuring antibodies. This antigen would have some defined role in tumorigenesis which as a corollary might also pertain to tumor escape from immune surveillance.

Objectives

The main objective of the research was to develop and define monoclonal antibodies (MAbs) to such an antigen for its identification and characterization, since such an antigen has been ill-defined. These MAbs would be used to assist in the purification of the antigen for further biochemical analysis and in particular to ascertain if naturally occurring antibodies could be demonstrated to this antigen and to address the PNA-reactivity by carbohydrate

inhibition testing. These MAbs would also be employed in biological assays on proliferation, differentiation, and/or activation to attempt to determine a biological function.

Approach and Overview of Results

With this in mind for the 167H fusion, PNA affinity enriched extracts of pooled human breast biopsy membranes were used as an immunogen. This thesis concerns the characterization of the antigen detected by the 167H.1 and 167H.4 MAbs which resulted from this fusion. Both appear to define a novel, 67 kilodalton, tumor associated glycoprotein expressed in 90 % of common, human adenocarcinomas which was subsequently characterized as a soluble AFP binding protein which also associates with the cell surface and appears to form part of the functional AFP receptor. Not only were isoforms of this receptor PNA reactive, but naturally occurring antibodies of both the IgM and IgG isotypes reactive with purified antigen were demonstrated. A potential role of this antigen as an anti-cellular senescence oncogene is suggested and a role of this antigen in the immunosuppression of the host is implied (autocrine and paracrine effects of the AFP-BP and/or AFP).

II. MATERIALS AND METHODS

A. PRODUCTION OF MONOCLONAL ANTIBODIES

Extraction and Immunization

Pellets containing breast cancer biopsy membranes were received from Dr. W. McBlain of the Hormone Receptor Laboratory, Edmonton. The breast cancer tissue had been suspended in a 10 mM Tris buffer containing the following: 12 mM thioglycolate, 10% glycerol, 10 mM sodium molybdate, 1.5 mM EDTA (pH 7.4), 12mM monothioglycerol, and after homogenization, was centrifuged at 200,000 G for 30 minutes. For the breast cancer cytosols, the supernatant had been removed from beneath the lipid layer. The pellets were pooled and extracted in 0.5% NP-40-0.01M Tris - (2mM) PMSF buffer (1 ml per pellet) for 45 minutes at 4^0C and centrifuged at 100,000 G. After removal of the top lipid layer, the supernatant was concentrated five fold by Amicon filtration. A 0.5 ml aliquot was added to 0.5 ml of 50% PNA-agarose in PBS (EY Labs) and incubated half an hour at 4^0C. After washing three times with cold PBS, the complex of PNA-agarose-antigen was injected i.p. into an RBF mouse. PNA-agarose was the sole adjuvant used (Morgan et al, 1984). Three days after the third, weekly injection, the spleen was removed and fused to the FOX-NY partner as described by Taggart and Samloff, 1983.

Fox-NY Fusion Method

The myeloma cell line, Fox-NY is doubly enzyme deficient for APRT and HPRT (adenosine phosphoribosyltransferase, and hypoxanthine phosphoribosyltransferase, respectively). Selection in a medium requiring APRT activity eliminates unfused APRT myeloma cells. However, because of the 8.12 translocation of the Robertsonian (8.12) 5Bnr mouse, whereby the APRT gene is genetically linked to the active heavy chain Ig locus, this also eliminates hybridomas which do not retain the heavy chain locus. This provides an advantage over the usual hybridoma fusion system (Taggart and Samloff, 1983). The mouse

was bled before taking the spleen. Spleen cells were isolated by gentle perfusion a 26-gauge needle and RPMI media in a petri dish. Contaminating RBC's were not removed in this procedure. Ten mls of cold medium containing 20% FCS were added to the isolated cells, centrifuged, resuspended and counted. Both the spleen cells and myeloma cells were then washed in media without FCS. The fusion was done at the ratio of 1:5 (myeloma:spleen cells) by adding 0.2 ml of PEG (30% polyethylene glycol, Sigma: 1300 to 1600 molecular weight) and gently resuspending the pellet. The fusion was left to proceed for 2 minutes and then was centrifuged at 800 rpm for 6 minutes. After centrifugation the pellet was loosened by gentle tapping and the addition of 5 mls of media. Then, 5 mls of media containing 20% FCS was added. The cells were centrifuged and resuspended in AAT media (30 mls per 1 x 10^7) and left in the hood for 1-2 hours. The cells were then distributed in 50 ul per well to which 3 x 10^4 per ml of macrophages (100 ul) had been added. (The macrophages were isolated from Balb/c by i.p. effusion of 5 mls of a 11.6% sterile sucrose solution.)

AAT Selection Media

The selection media contained 15% FCS (heat-inactivated, Gibco) in RPMI, 7.5 x 10^{-5} adenine, 8 x 10^{-7} aminopterin and 1.6 x 10^{-5} thymidine and supplemented with glucose, oxalacetic acid, sodium pyruvate, insulin and L-glutamate. The antibiotic was penicillin (100 u/ml) and streptomycin (100 ug/ml) from Gibco.

Screening

The screening was performed on PNA-agarose affinity purified material and was negatively selected on human serum albumin (HSA, Sigma) by ELISA, always by an overnight incubation when supernatants were employed. The hybridomas secreting selected MAbs were cloned twice by limiting dilution.

Affinity Purification of PNA Glycoproteins

For the affinity purification, after extraction of thirty membrane pellet equivalents, the material was placed on 20 mls of PNA-agarose, incubated for half an hour at 4^0C. After washing five times with PBS, 20 mls of elution buffer was added (5 mls of glacial acetic acid and 8.76 g of NaCl to one liter). This was then dialysed against PBS and diluted 1/10 when added to plates.

Freezing of Cells

Chosen clones were prepared for freezing by adding slowly with shaking a 30% FCS-10% DMSO-RPMI media to 1 x 10^6 cells on ice. After 24 hours at -70^0C, these were transferred to liquid nitrogen.

B. ELISA

Extracts/Antigen

Antigen (1 ug/well), pooled tumor extracts (diluted 1/5 in PBS), or a lung metastatic breast carcinoma pleural effusion (Ptnt 89, diluted 1/30 in PBS) was added in a volume of 50 ul to Nunclon ELISA plates overnight at 4^0C. The plates were blocked in 2% gelatin-PBS (Biorad) for one hour at room temperature. Unless otherwise stated, the ascites was diluted 1/300 in 1% gelatin-PBS (hybridoma supernatants were not diluted) and allowed to react for 2 hours at room temperature. After washing 3-5 times with PBS, the HRP labeled second antibody, (KPL-goat anti-mouse G/M-human serum absorbed) was used at 1/200 in 1% gelatin-PBS and incubated 1-2 hours at room temperature. After the addition of 100 ul of ABTS-H_2O_2 (1:1, KPL) the plates were read at 30 minutes at 405 nm with subtraction at 540 nm.

Pure glycophorin and HSA were purchased from Sigma, pure AFP was purchased from Dr. Wu, and the synthetic TF antigens coupled to HSA (glycoprotein type) or synthetic TF-like antigens coupled to HSA (ceramide, glycolipid type) were kindly provided by Biomira

Inc. (Edmonton, Alberta, Canada). Epiglycanin (epi) was a kind gift of Dr. Carina Henningsson.

Antibodies

A rabbit anti-human AFP antisera, or later a murine monoclonal antibody to human AFP (IgG1) was purchased from Calbiochem.

Most of the antibodies used for these studies were available through the Longenecker laboratory or available in the Department of Immunology. The IgM negative control antibodies used included Y5781.4, CH4, CH5 or later, NS1 Clone 20 (a kind gift of Dr. Linda Pilarski). The 167H.3 MAb produced in the same fusion as the 167H.1 and 167H.4 MAbs was often used as a IgM control antibody. This MAb had reactivity with PBMC but not with thymocytes. The fine specificity of the 167H.3 MAb was not determined. The 167H.2 MAb, also produced in the 167H fusion was found to react with synthetic Tn antigen, and being also an IgM, was often employed as a control antibody. Preliminary evidence suggested although this MAb might also react with AFP-BP isoforms, it did not inhibit the binding of AFP and its reactivity was not restricted to the AFP-BP. It seemed to also identify high molecular weight mucins of breast cancers. Since the 167H.2 clone was lost, only limited amounts of original ascites was available for studies. Control IgG monoclonal antibodies used were the 153H.1 MAb which was specific for the PNA lectin, or F23.1 (IgG2a) specific for a murine T cell receptor. Other antibodies employed for secondary studies were 7H.3 (IgG) which is against human Class II MHC antigens, 155H.7 (IgG) which was produced to synthetic (ceramide-type) TF antigens, 9H.3 (IgG) which reacts with human B_2m, and the 49H.8 MAb (IgM) which reacts with the red cell cryptic TF antigen.

Cell Lines

All cell lines were maintained in 10%-FCS (Gibco), RPMI media (Gibco) supplemented with 2 mM L-glutamine and 10 ug/ml penicillin-streptomycin with the addition of 2-ME for murine cell lines. All cell lines were routinely tested for lack of mycoplasma

infection. All experiments were performed with mycoplasma-free cell lines.

Murine Bone Marrow Macrophages

Bone marrow macrophages were derived from DBA/2J female mice as previously described (Lee et al, 1980). Briefly, a million bone marrow cells were cultured for 5 days in 25 mls of Dulbecco's modified minimal essential medium, containing 10 % L-cell conditioned medium (LCM), 18 % horse serum and 2 % FCS. Under these conditions bone marrow cells grow exponentially to form colonies of pure macrophages. Bone marrow macrophages were washed three times in RPMI prior to subsequent manipulations to remove any remaining LCM.

Cellular ELISA

Nunc micro-ELISA plates were coated with 100 uls of a 50 ug/ml solution of poly-L-lysine in PBS at 4^0C overnight. The plates were washed once with 200 uls of PBS per well. 100 uls of a 0.1% glutaraldehyde in 0.1 M $NaHCO_3$ was added and incubated for 3 hours at room temperature. After another wash, 1 x 10^5 cells in 50 uls of PBS was added and centrifuged at 1100 rpm for 4 minutes. After flicking off the supernatant, the cells were gently fixed in 200 uls of 0.05% glutaraldehyde in PBS solution for 3-5 minutes. The plates were blocked and treated as above.

C. IMMUNOHISTOLOGY

Frozen sections were immunohistologically stained using either supernatants or diluted hybridoma ascites (1/200 - 1/500) by the avidin biotin procedure already described (Longenecker et al, 1984). Briefly, the first antibody diluted in 1 % BSA-PBS was incubated for one hour on 6 microns thick frozen sections which were kept in PBS. The sections were then washed 5 minutes in PBS and incubated with a biotinylated second antibody (Vector Labs) for 30 minutes. After another 5 minute wash, the slides were incubated with the avidin ABC reagent (Vector Labs) for 20 minutes. After a further wash of 5 minutes the slides were

developed in a 0.5% H_2O_2 - DAB solution (60 mg of DAB to 100 mls of PBS) for 5 minutes. For the counterstain, after a wash in distilled water, the slides were immersed in Harris's Hematoxylin for 30 seconds, differentiated in 0.01% Acid-Alcohol (a few dips) and blued in 1% ammonium-water. The sections were dehydrated as per the usual alcohol treatment for tissue sections, cleared in xylene and mounted in permount.

D. ISOTYPING OF MAbs

A murine isotyping kit was purchased from ZYMED Laboratories Inc. (California). Hybridoma supernatants collected for 24 hours in serum-free media were incubated overnight (100 ul) onto NUNCLON 96 well plates. After blocking with 1 % BSA-PBS (KPL) rabbit anti-mouse heavy chain specific antisera (one drop per well) was added and incubated for one hour. After washing three times with PBS, goat-anti-rabbit IgG (peroxidase conjugate) was diluted 1/50 and 100 ul was added per well. After one hour, the plates were again washed and developed using the KPL ABTS: peroxide reagents.

E. PREPARATION OF CELLS FOR FACS ANALYSIS

Cell Lines

The adherent LoVo cells were isolated by EDTA removal, (0.02 % in PBS). These or the non-adherent TA3-Ha cells or other cells were washed three times in RPMI and incubated at 37^0C for 2 hours to deplete the cells of FCS. The cells were then washed with PBS-azide (0.02%) and incubated 2 hours at room temperature (1 x 10^6/V-bottom well), with 100 ul of 1/300 ascites diluted in 1% BSA-PBS-azide. The cells were washed three times with 150 ul of PBS-azide and incubated for 1 hour with 100 ul of fluorescein isothiocyanate labeled, goat anti-mouse G/M/A (FAb), (Cappel Labs), at 1/20 in 1%-BSA-PBS-azide. After washing twice, 150 ul of a 4% formaldehyde-PBS was added to each well and later FACS analysis was performed. For the PNA positive control, PNA (1 mg/ml, E-Y Labs) was diluted 1/100 and was developed using 153H.1, an anti-PNA MAb produced in this laboratory.

PBMC, T Cells, or Adherent Cells

Human T cell isolation columns were purchased from Sci-Can Diagnostics (Edmonton). PBMC's were isolated from buffy coats (Canadian Red Cross, Blood Transfusion Service, Edmonton) as follows. The buffy coat was centrifuged at 1600 rpm for 10 minutes. The top layer of white blood cells was resuspended in 5 mls of RPMI media and layered onto 5 mls of Lymphocyte Separation Media (Litten Bionetics). These tubes were centrifuged at 1800 rpm in Falcon tubes for 20 minutes, and washed three times with RPMI media. The cells were counted in trypan blue and 5×10^7 cells were resuspended in 2 mls of RPMI for isolation of T cells, or 1×10^7 per ml to isolate adherent cells. For adherent cells, see method under Human Adherent Cell Tumor Cytotoxicity Assay.

The T cell separation column operates on the principle that goat anti-human Ig coated beads will bind B cells via surface Ig allowing T cells and macrophages to pass through the column. Quality control of these columns indicates that less than 1 % of filtrate cells are B cells and that the LPS response (specific for B cells) is neglible. The column was prepared by first washing the column with about 20 mls of RPMI media. Goat anti-human Ig (0.5 mls) was diluted to 2 mls with media, and was incubated on the beads for one hour at room temperature. After washing the column with about 20 mls of media, the flow rate was adjusted to about 6 drops per minute and 5×10^7 PBMC's were run over the column in media. The first 20 mls was collected and washed. Typically about 80% of the total number of cells applied were recovered suggesting that all or most of the T cells were isolated. For the activation of T cells, a 1 mg/ml solution of CON-A (Sigma) was diluted 1/50 in RPMI-5% AB serum containing 1×10^6 cells/ml suspension. A separate aliquot of cells was made omitting the CON-A. Both activated and non-activated cells were incubated for 72 hours at 37^0C then harvested and prepared for FACS analysis as outlined above.

Thymocyte Isolation and Negative Depletions

Human thymocytes were prepared from children undergoing cardiac surgery, and the relevant subpopulations depleted according to the protocols previously reported (Pilarski et al,

1989). Briefly, the tissue was chopped in sterile 10%-FCS-RPMI containing DNAse (0.5 mg/ml) and no phenol red, and the cells isolated after sieving. The thymocyte population was isolated by Ficoll-Paque (25 minutes at 1800 rpm) and then washed twice in cold FCS-RPMI media.

To deplete thymocytes, either the FMC 71 (CD45RA, p220) or the UCHL-1 (CD45RO, p180) and FMC 63 to deplete B cells, were added to thymocytes and incubated for 20 minutes on ice. Alternatively, anti-CD3, CD4, and CD8 antibodies were used for the depletion step to enrich for multinegative thymocytes. These cells were washed to remove unbound antibody, and the antibody bound cells depleted with four rounds of Dynabead treatment with a magnet. The Dynabeads were coupled with anti-mouse Ig (Dynal, Oslo, Norway; 0.2 ml Dynabeads/10^7 cells.) The efficiency of the depletions was separately assessed by FACS analysis with the relevant antibodies (data not shown). For the multinegative thymocyte populations it was necessary to remove contaminating red cells. For this Lymphocyte Separation Media (Litten Bionetics) was used by centrigugation at 1600 rpm for 20 minutes, as a final step prior to culture or analysis.

Principle of FACS Analysis

In brief, the cells are tagged with the primary antibody and developed with a FITC labeled second antibody. By FACS analysis the relative mean fluorescence per cell can be electronically gated and once this is set, a percentage of the total cell population fitting this criteria can be obtained. FITC has a maximal excitation wavelength at 428 nm, and when the laser excites the fluorochrome, fluorescence occurs at a higher wavelength of 525 nm. This shift allows the use of selective optical filters to eliminate unwanted signals. The light signals are converted to electrical signals by photodetectors and these in turn are processed by a computer.

For the analysis, cells are individually passed through a small orifice, the volume of which displaces voltage. This provides a reading of size which is simultaneously processed by the computer with the corresponding fluorescence, on a cell to cell basis. Thus,

subpopulations of different sizes can also be computated for their fluorescence. Background fluorescence can be gated out by using the appropriate negative control sample. The FACS analysis is sensitive to about 1 % of the population.

F. PURIFICATION OF MONOCLONAL ANTIBODIES

Ascites were produced in Balb/c mice as previously described (Longenecker et al, 1984), whereby the mice were pristane primed (0.5 ml) at least two weeks prior and given 500 rads twenty-four hours before innoculating 1-10 x 10^6 hybridoma cells i.p.. Monoclonal antibodies were purified using the one-step hydroxylapatite method of Stanker et al (1985). About 50 mls of a 50 % hydroxylapatite-agarose (Ultragel-HA, LKB) suspension was placed in a general purpose column (Biorad) and was connected to a LKB fraction collector. The column was washed with Solution A. Solution A contained 0.709 g of Na_2HPO_4 (.005 M) and .689 g of NaH_2PO_4 (.005 M) per litre. The filtered ascites (15 mls) was diluted 1/2 with Solution A and applied to the column. The flow rate was adjusted to 60 mls an hour. At the point where the recorder returned to background levels, a linear gradient was set up using Solution A and Solution B. Solution B contained 21.3 g of Na_2HPO_4 (.150 M) and 20.7 g of NaH_2PO_4 (.150 M) per litre. The fractions were tested by ELISA for the presence of MAb, and the corresponding fractions were dialysed and concentrated by Amicon filtration to 1 mg/ml. Purification was checked on reducing SDS-PAGE gels (data not shown). Unless otherwise stated, the purified antibodies were used at a 1/100 dilution.

G. SDS-PAGE AND PROTEIN TRANSBLOTTING

Proteins can be separated based mostly on size by SDS-PAGE, since SDS imparts a uniform net negative charge. In an electric field, the migration becomes independent of charge and mostly reflects size although aberrations in migration can be due to sialic acid content, phosphorylation levels, percent carbohydrate or if an excess of disulphide bonds exists for a given protein. Since the protein is negatively charged, it migrates towards the anode, or to the bottom of the gel. Smaller proteins migrate faster.

Protein transblotting (often referred to as western blotting) offers an easy analysis for identification of antigens after separation either on size or charge. In this procedure, after separation, the proteins are transferred to a solid matrix, often nitrocellulose, blocked and then probed with antibodies. The bands are revealed by essentially an ELISA technique but where the colored product is insoluble. Transfer to nitrocellulose from SDS-PAGE involves either heat (Phast-Gel) or electrophoretic transfer (Biorad) while transfer from IEF gels (which is a softer matrix) is performed simply in an humidity chamber by placing the nitrocellulose over the gel and waiting about 45 minutes.

Western blotting may give identical results to the more classical immunoprecipitation technique or may complement this alternative. This is because immunoprecipitation can bring down an antigen complex provided the antibody used does not inhibit the complex formation. In general, western blotting may not work on reduced transblots while the classical immunoprecipitation is superior for reduced sample analysis.

Biorad, Macro Procedure

This technique was performed according to manufacturer's instructions (Biorad) using the Laemmli buffer system. Solutions were made as follows.

Acrylamide

29.2 g acrylamide

0.8 g N'N'-BIS methylene acrylamide

Dilute to 100 ml with ddH_2O and filter and store in the dark.

1.5 M Tris-Cl, pH 8.8

18.15 g Tris base

50 ml ddH_2O

Adjust to pH 8.8 with 1 N HCl.

Dilute to 100 ml with ddH_2O.

0.5 M Tris-Cl, pH 6.8

3.0 g Tris base

50 ml ddH_2O

Adjust to pH 6.8 with 1 N HCl

Dilute to 50 ml with ddH_2O

Separating 3-15% Gel Preparation:

For the 3% Gel:

19.14 mls ddH_2O

7.5 mls 1.5 M Tris-Cl

3.00 mls Acrylamide-BIS

0.3 mls 10% SDS

and after degassing:

50 ul 10% (w/v) Ammonium Sulphate

10 ul Temed

For the 15% Gel:

4.14 mls ddH_2O

7.5 mls 1.5 M Tris-Cl

15.00 mls Acrylamide-BIS

300 ul 10% (w/v) SDS

and after degassing:

50 ul 10% (w/v) Ammonium Sulphate

10 ul Temed

3.0 mls glycerol

Stacking Gel Preparation

6.1 mls ddH_2O

2.5 mls 0.5 M Tris-Cl, pH 6.8

100 ul 10% (w/v) SDS

1.3 mls Acrylamide-BIS

50 ul 10% (w/v) Ammonium Sulphate

10 ul Temed

Electrode Buffer

12.0 g Tris base

57.6 g Glycine

4.0 g SDS

Dilute to 4 litres with ddH_2O

Sample Buffer

4.0 mls ddH_2O

1.0 ml 0.5 M Tris-Cl pH 6.8

800 ul glycerol

1.6 mls 10% (w/v) SDS

0.2 ml 0.05% (w/v) Bromophenol Blue

and optionally, 400 ul 2-ME

For the run, 50 ul of sample diluted at least 1/4 with sample buffer was applied per lane. The gels were run at 20 mA (for 2 gels) until the tracking dye reached the separating gel. Then the gels were run at 250 V for several hours with water cooling.

Coomassie Blue Staining

The gels were stained for 15 minutes in 10% acetic acid, 25% isopropyl alcohol containing 1 g/litre of R-250 Coomassie Brillant Blue (Biorad) and destained overnight in 10% acetic acid containing 10% methanol.

Protein Transblotting

The transblot apparatus was set up according to manufacturer's instructions (Biorad). The procedure was modified according to the following: transblot buffer was sodium phosphate (30 mM), blotting was performed for 4 hours at 0.9 amps, no Tween was used in the PBS washes, and the blocking reagent used was 2 % gelatin-PBS. Purified MAbs were used at 1/100, and development was with the BCIP/NBT-Alkaline Phosphatase system (Biorad) where the second antibody (goat-anti-mouse IgG, Biorad) was used at 1/600 in order to detect both IgG and IgM MAbs. High and low molecular weight standards (Biorad) were also transblotted and stained with Amido Black (Biorad). Control 153H.1 is an anti-PNA MAb. Control MAb 7H.3 is an IgG MAb reactive against a common determinant of human Class II DR antigens (MacLean et al, 1982).

Transblot Buffer

21.45 g of Na_2HPO_4-7H2O and 5.52 g of NaH_2PO4 was added to 4 litres of ddH_2O (pH 7.4).

Carbonate Buffer for Alkaline Phosphatase Development

16.8 g of $NaHCO_3$ plus .406 g of $MgCl_2$-$6H_2O$ was diluted to 800 mls with ddH_2O. After the pH was adjusted to 9.8 with NaOH, this solution was made to a litre.

BCIP/NBT Color Development Solution

A 70 % DMF (N,N-dimethylformamide, Anachemia) was prepared by mixing 0.7 ml of DMF with 0.3 ml of ddH_2O and 30 mg of NBT (Biorad) was dissolved in this. In a separate tube, 15 mg of BCIP was added to 1 ml of DMF. These solutions (prepared fresh) were added to 100 mls of carbonate buffer just prior to use.

HRP Color Development Solution

60 mg of HRP Color Development Reagent (Biorad) was added to 20 mls of cold methanol. Just prior to use 60 ul of cold 30 % H_2O_2 was added to 100 mls of PBS. Since this stain has a normal pH, a single nitrocellulose sheet can be reprocessed several times without denaturing the antigen. This is unlike the alkaline phosphatase system in which the epitopes examined here seem to be sensitive to the extreme pH.

Amido Black Staining Protocol

Amido Black Stain

0.1% (w/v) in 45% methanol and 10% acetic acid.

Destain

90% ethanol in 2% acetic acid.

The nitrocellulose strip was flooded in 6% TCA. The strip was then submerged in the amido black for about 30 seconds until it was uniformly a pale blue. After a brief rinse in ddH_2O the strip was destained twice for about 30 seconds in a volume of 200 mls. A ddH_2O wash followed this. The strip was blotted dry and wedged flat to dry.

Pharmacia, Micro Procedure

Either precast pI 3-9 isolectric focussing gels were used or 10-15% SDS-PAGE gels. For the former, transblotting was done by simple diffusion in a humidity chamber for one hour at room temperature. The gel was then stained with the silver technique for the IEF gels using the Phast-Gel apparatus and the protocol of the manufacturer (Pharmacia). Silver staining reagents employed were from Biorad except the glutaraldehyde was from Fisher Scientific, Canada. For IEF gels the standards of Pharmacia were used by diluting the material with 50 ul of ddH_2O. Routinely, the gels were loaded from the center application wells.

The Phast-Gel system has many advantages including time, reproducibility, and small sample size (1 ul per well). Isoelectric focussing, under non-denaturing conditions (the conditions employed here), separates proteins on their net charge but also based on its conformation, which may then alter slightly the migration of the proteins. Complexes, are not likely to dissociate.

H. METHIONINE LABELING AND IMMUNOPRECIPTIATION

Cells which were washed and resuspended in methionine free media (DMEM) supplemented with 10% dialysed FCS, were adjusted to 5 x 10^7/ml and 1 millicurie of ^{35}S-methionine (NEN Canada) was added per 1 x 10^8 cells and labeled overnight. After washing the cells five times in complete media, the cells were extracted in the extraction buffer (same as for extraction of breast biopsy membranes) (one ml per 1 x 10^8), and precleared three times on anti-mouse IgM-Agarose (200 ul). To a 300 ul aliquot was added 100 ul of purified MAb (1 mg/ml) for one hour. This was then followed by 200 ul of a 50% solution of anti-mouse IgM-Sepharose (Sigma) and tumbled overnight at 4^0C. This was then washed five times with PBS and the retained proteins eluted with SDS-PAGE sample buffer with or without reducing agents. After the gel electrophoresis, the gels were impregnated with enhance and this fluor precipitated with cold 5 % acetic acid-5% glycerol. The gels were exposed for 5 to 7 days at -70^0C for the autoradiography in a Kodak X-Omatic cassette with fine intensifying screens.

I. TREATMENT OF ANTIGEN

Sodium Borohydride Treatment of Antigen

In order to determine if the epitopes recognized by these MAbs involved carbohydrates, antigen was treated with alkali-borohydride (Sekine et al, 1985). Briefly, 5 ml of Ptnt 89 was incubated for 24 hours at 37^0C with 5 mls of 0.2 N NaOH containing 2 M $NaBH_4$ (Sigma). The reaction was neutralized with 750 ul of glacial acetic acid and dialysed

against several changes of PBS in the cold. After adding the treated antigen to ELISA plates (at 1/15 dilution in PBS), the plates were treated with 6 M guanidine-HCl for 30 minutes, followed by washing five times in PBS before blocking as per the normal ELISA.

Neuraminidase Treatment

For neuraminidase treatment of antigen, equal volumes of neuraminidase (Sigma), diluted to 1 unit/ml in PBS, and Ptnt 89 were incubated for 2 hours at 37^0C. This was immediately used or the sample frozen until use, for the western blots. Alternatively antigen coated plates, after blocking, were treated with 2.5 units/10 mls of PBS neuraminidase solution for 2 hours at 37^0C and then washed 5 times prior to conducting the ELISA.

Mild Periodate Treatment

Antigen (PE, 1/30) was also treated with mild periodate within the ELISA plates according to the method of Woodward et al, 1985, to determine if the MAbs detected at least in part, carbohydrate determinants. Traditionally, susceptibility to mild periodate treatment as described by Woodward et al, is considered indicative of the carbohydrate nature of antigens, although it may not work on all carbohydrate structures (Feizi et al, 1987b). This was performed by rinsing the plates with 50 mM sodium acetate buffer (pH 4.5) after blocking. The plates were then exposed to 10 mM m-periodate (Sigma) in 50 mM sodium acetate buffer for 1 hour at room temperature in the dark. After a rinse in acetate buffer, the plates were then incubated with 1 % glycine in PBS for 30 minutes to block the aldehyde groups generated by the periodate oxidation. This prevents non-specific cross-linking of antibody to antigen. The plates are washed 5 times with PBS and then processed as usual for the ELISA.

J. PREPARATION OF RABBIT ANTISERA TO CRUDE AFP-BP

It was desirable to have a rabbit antisera to the AFP-BP since it was likely both the 167H.1 and 167H.4 MAbs detect in part, carbohydrates. Such an antisera would be useful for the screening of lambda gt11 expression vector library in order to pull out the corresponding

cDNA clone, for checking the quality of the purification, and for immunoprecipitation studies. As well, a fourth very important use of the rabbit antisera was the demonstration that *E. coli* absorbed antiserum lost its reactivity with certain isoforms of the AFP-BP suggesting cross-reactions of the AFP-BP with gram negative bacteria, most likely carbohydrates.

Immunization

A New Zealand White rabbit was obtained from the Health Sciences Laboratory Animal Services. 1.5 mls of HTP purified AFP-BP containing 0.9 mg of total protein (derived from Ptnt 89), was added to one vial of Ribi (Ribi Immunochem Research Inc., Hamilton, Montana, U.S.A.). For each immunization once every 10 days (repeated twice), a 0.5 ml aliquot of the antigen-Ribi was mixed with 0.5 ml of PNA-Agarose (50 % solution, E-Y Labs) and injected intramuscularily. Approximately 300 ug of antigen per 170 ug of Ribi was injected per immunization. The adjuvants employed were PNA-Agarose, MPL (monophosphoryl lipid A from *S. minnesota*) and TDM (trehalose dimycolate from *M. phlei*). Prior to the initiation of the immunization the rabbit was bled 30 mls of whole blood.

After each immunization the antisera was titrated, aliquoted and stored at -70^0C. The second antibody used here was a goat anti-rabbit Alkaline Phosphatase labeled antisera (Biorad). The development solution for this antibody in the ELISA was one tablet containing 5 mg of p-nitrophenyl phosphate (disodium, Sigma) to 5 mls of 10 % diethanolamine buffer, pH 9.8.

K. PROLIFERATION ASSAYS AND EFFECTS OF MAbs OR AFP

Purification of AFP

Human AFP was isolated from Ptnt 89 by chromatography on an HTP column using the methods for MAb purification (see Section F and Section M). The pre-albumin peak, shown to contain mostly AFP but some AFP-BP, is referred to "crude AFP". For the binding

inhibition studies of the 167H.1 and 167H.4 MAbs on a breast cancer cytosol, 9250, the AFP was further purified on a Con-A column (see Section O) and demonstrated not to contain any AFP-BP.

MTT Assay for Cell Survival and Proliferation

MTT is a pale yellow substrate that is cleaved by living cells to yield a dark purple formazan product. This process requires active mitochondria, and even freshly dead cells do not cleave significant amounts of MTT. The colorimetric assay can be used for cytotoxicity assays or for proliferation. This assay is sensitive to detect about 200 to 50,000 cells per well. The acid/alcohol is made by adding 4 mls of 1 N HCl to 100 mls 2-propanol (isopropanol). 5 mg/ml of MTT (Sigma) in PBS is filter sterilized and stored in the dark. The assay is set up as follows. First each cell line is titrated to standardize the assay so as the reading at 595 nm is an absorbance in between .2 to .4. Typically the optimum number of cells per well for a cell line was 1-5 x 10^4 and to study the effects of the MAbs or antigens on proliferation the 96 well plates (Linbro, flat-bottom) were incubated for 48 hours before adding 10 ul of the stock MTT per well. After mixing, the plates were then incubated at 37^0C for four hours for cleavage to occur (or until purple precipitates were detected on the bottom of the wells). 150 ul of the acid/alcohol solution are added to the 110 ul already present in the wells. The plates are immediately read at 595 nm with the first column serving as a control (no cells, media alone) from which the background is subtracted.

Fluorescent Staining of Non-Viable Cells

Propidium iodide and/or FITC (Sigma) were diluted to 0.5 mg/ml in PBS and sterile filtered with a 45 micron filter (22 micron filters absorb significant amounts of fluorochrome) and these stock solutions were frozen. For staining of dead cells, 10 ul of either or both were added to 100 ul of cells which had been placed in 96 well flat bottom plates and prewarmed at 37^0C for at least 10 minutes. The wells were not mixed. Immediately after incubation at 37^0C, the plates were washed three times with cold PBS (no Ca++ or Mg++) in the cold.

Thereupon the cells were fixed in cold PBS containing 0.02 % sodium azide and 1 % formalin and subsequently analysed by FACSCAN.

Although propidium iodide (PI) gives a good correlation to trypan blue exclusion (Sasaki \DT et al, 1987), it was found that staining with both fluorochromes gave more sensitive and meaningful results. For example, PI appears to stain dead cells but also slightly smaller cellular debris. FITC does not appear to stain the latter, perhaps because stained proteins might leak out through the larger perforations. On the other hand, PI does not appear to stain cells which are committed to cell death and are in the process of "committing suicide" but which are not yet dead (as judged by trypan blue). In this regard the staining by FITC of dying cells but which are not yet dead, provides a more practical approach to monitoring the death sequence. It should be mentioned that live cells can exhibit FITC uptake, but it is the relative increase in staining which is taken to represent the population committed to cell death. Thus, double negative populations (low PI or FITC uptake) are considered the viable population, PI single positives and double positives are considered the dead population, and single positive FITC (relative) are considered to be those committed to cell death but which are not yet dead. Kinetic studies on thymocytes suggests that single positive FITC (PI negative) cells still incorporate ^{3}H-thymidine until the cells reach a stage where PI is taken up.

Thymidine Incorporation

Cell cultures were pulsed with 1 uCi ^{3}H-thymidine (specific activity was 20 Ci/mM, NEN, Ontario) per well for overnight. Incorporation of the radiolabeled thymidine was measured by a standard liquid scintillation counting technique after harvesting cells onto glass fiber filters using an automatic titertech cell harvester (Flow Labs). Adherent cells such as LoVo were first exposed to 200 ul of 1 N NaOH before harvesting. The dried filters were added to scintillation vials containing 2-3 mls of toluene with 1.38 g of omnifluor per litre and counted in a RackBeta 1218 (LKB, Wallac, Finland) counter. Thymidine uptake is expressed as mean counts per minute (cpm) +/- standard error (s.e.) from triplicate wells.

Mixed Lymphocyte Reaction

Stimulator cells were irradiated 2000 rads prior to culture. 1 x 10^5 stimulator cells were added to the same number of responder cells in 5 % AB serum - RPMI in round bottom micro-titre wells (Costar). After 3 days the cultures were pulsed and harvested as above for the thymidine incorporation. Murine spleen cells were isolated as per the isolation technique used for human PBL's except the cells were centrifuged at 1600 rpm for 20 minutes when layered onto the Lymphocyte Separation Media.

Human Adherent Cell Tumor Cytotoxicity Assay

PBL were isolated as previously described and 2 x 10^7 cells in 100 ul of 5% FCS-RPMI were plated per well in round bottom sterile 96 well plates (Corning) and incubated for 1 to 2 hours at 37^0C according to the method of Philip (1988). The wells were then washed three times with warmed media containing 5% FCS. It has been previously shown that the adherent population so obtained contains about 90 % monocytes and the remainder a mixed population of NK or LAK precursor cells and possibly other unknown cells. The adherent cells were activated either by 100 ul of a 24 hour MLR supernatant, or 100 ul of media containing 20 U of rIL-2 (Cellular Products Inc.) for 48 hours with or without added proteins. The constitutive response was without added activators. BW5147 a murine T cell fusion partner which does not express the AFP-BP or 68.8 a T cell hybridoma provided by Dr. Michel Boyer, Edmonton, were labeled overnight with ^{51}Cr with 100 uCi per million cells. Prior to the 18 hour cytotoxicity assay, labelled cells were gently washed twice, incubated at 37^0C for half an hour then washed two more times and resuspended to two million cells per ml in 5% FCS-RPMI. Then 10 ul of target cells was added per well which was estimated to contain approximately two million adherent cells per well giving approximately a 20:1 effector/target ratio. Results are expressed as the results of triplicates or quadruplicates and specific release was calculated by subtracting the spontaneous release from the maximal and test release and calculating the percentage of the total.

L. LABELING OF PROTEINS

Radioactive Iodine Labeling

^{125}I labeling of proteins was performed by the chloramine T method of Hunter, 1978. 1 millicurie of radiolabeled sodium iodide (Edmonton Radiopharmaceutical Centre) was added to 200 ug solution of the purified protein in PBS. 50 ul of chloramine T (2 mg/ml) was added to the mixture and the oxidation reaction allowed to continue for 2-5 minutes on ice. The reaction was stopped by the addition of 50 ul of sodium metabisulfite (6 mg/ml). 10 ul of 1 % potassium iodide was added and the solution passed over a small 5 ml G-50 gel filtration column (in PBS) to remove the free iodine.

Biotinylation of Proteins

Biotin (NHSLC-Biotin) was purchased from Pierce through Terochem and the Strept-Avidin HRP Complex was ordered from Amersham. Proteins (1 mg/ml) were labeled with the water soluble biotin by the procedure of Imam et al, 1986. For 4 mls of protein in PBS, 1 ml of 0.2 M $NaHCO_3$ buffer containing 0.15 M KCl (pH 8.8) and 100 ul of biotin (40 mg/ml) in PBS were added. After shaking for 15 minutes at room temperature 0.5 ml of 1 M NH_4Cl buffer (pH 6.0) was added to stop the reaction. This solution was then dialysed overnight against PBS. After titration of the probes by ELISA the labeled proteins were aliquoted and stored at -70^0C.

For the development of biotinylated probes, after washing, strept-avidin-HRP complex was diluted 1/750 in 1 % BSA-PBS and incubated one hour at 37^0C. After washing three times in PBS, the plates were developed with ABTS and H_2O_2 as per the usual ELISA technique.

M. PURIFICATION OF AFP-BP

Two-phase, Non-denaturing Procedure

The purification procedure was designed to be simple, and to be non-denaturing. A two-phase procedure was implimented so as to recover AFP as well as AFP-BP. The first step is the chromatography of Ptnt 89 or Tbm on the HTP column using the same procedure as for the monoclonal antibody purification. The second step is the further resolution of the HTP peaks on a Mono Q column (5/5) using the Pharmacia FPLC system. Buffer A was a 20 mM Tris (pH 7.6) and Buffer B was the same but with 1 M NaCl. The flow rate was 0.5 ml/min, the pressure was 1.5 MPa, the detector was set at 280 nm, the sensitivity was 0.1 absorbance, and the chart speed was set to 0.5 cm/minute.

Anti-AFP-BP Affinity Column

Purified 167H.4 MAb (10 mgs), was coupled to cyanogen-bromide activated Sepharose-4B (Pharmacia) with 0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.3. Blocking of excess activated groups was carried out with 0.2 M glycine, pH 8.0 and uncoupled ligand was removed with 0.1 M sodium acetate, 0.5 M NaCl, pH 4.0. The column was equilibriated with Ca^{++}/Mg^{++} free PBS and 20 mls PE was applied to the column. Bound AFP-BP was eluted with 0.1 M glycine, pH 2.5. This was neutralized with 1 N NaOH. This was dialysed against PBS and concentrated by Amicon filtration and an estimate of its concentration was measured at A_{280}.

N. AFP BINDING INHIBITION EXPERIMENTS

Purification of AFP

Anti-human AFP (a generous gift of Dr. F. Lorscheider) was coupled to CNBr activated sepharose (Pharmacia), and used to purify AFP from cord serum or from serum-free supernatants of a human hepatoma cell line (kindly provided by Dr. T. Tamaoki),

for the earlier experiments. As the affinity column was accidently ruined (Dr. Ricardo Moro, personal communication) for later experiments AFP was purified by its ability to bind to the AFP-BP (HTP-Con-A, 2-step purification, see later).

Cell Lines

Ichikawa cells, a human T-cell leukemia and TA3-Ha, a murine mammary adenocarcinoma were selected on their ability to specifically bind FITC-labelled AFP. These cell lines were routinely cultured in 10 % FCS-RPMI and in order to deplete the cells of FCS and bovine AFP, were washed 3 times in RPMI and incubated for 2 hours at 37^0C. Initial experiments showed that the mild glutaraldehyde fixation used in the fixed cell ELISA did not disrupt the specific binding of AFP to live cells found at 4^0C. The fixed cells (1×10^5 per well) were incubated overnight with the 167H fusion positive supernatants (100 ul) or with 15 % FCS-RPMI medium as a negative control. The wells were washed 3 times with PBS and 70 ul of a 1-3 ug/ml solution of ^{125}I-AFP in 1% Gel-PBS with or without 1 mg/ml cold AFP. This was incubated for 1 hour then washed 3 times with PBS. The counts present per well were isolated by adding 100 ul of 1 N NaOH and collecting the solubilized cells and counted in a LKB gamma counter. For the initial screening, the supernatants were tested in duplicate and controls were assayed in quadruplicate. Results are expressed as the percentage of the ^{125}I-AFP binding inhibition by the MAb supernatants compared to controls according to the following formula:

% Inhibition = 1.0 - (MAb cpm - cold AFP cpm)
/ (Total cpm - cold AFP cpm)

Western Blot with Radiolabeled AFP

5 % PAGE (non-denaturing) analysis was used for this since SDS-PAGE analysis did not appear to work. Ptnt 89, a pleural effusion of a lung metastatic mammary carcinoma was

used as a source of the putative receptor. Using the macro technique of Biorad, adjacent wells were probed with the 167H.1 and 167H.4 MAbs as well as with ^{125}I-AFP (200 ug/50 mls of 1 % Gel-PBS). After a 2 hour incubation the excess radiolabel was washed off for 5 hours with rotation. The dried nitrocellulose was exposed for the autoradiography for 3 days. The bands were visualized by densitomer scanning on a CAMAG electrophoresis scanner.

O. A NEW METHOD TO PURIFY AFP

Samples of Ptnt 89 (a pleural effusion of a lung metastatic breast cancer) were run on an HTP column as described for the purification of the AFP-BP. The HTP fraction corresponding to the pre-albumin peak was affinity purified on a Con-A-sepharose column (Sigma). For this 20 mls of the AFP/AFP-BP HTP eluate containing approximately 1 mg/ml, was added to 30 mls of packed Con-A matrix which had been washed three times with Gey's Balanced Salt Solution (GBSS) which contains Ca^{++} and Mg^{++}. This was mixed overnight at 4^0C. The following day the Con-A column was with GBSS (five times, 20 mls) and 20 mls of 0.3 M KCl in GBSS (to elute bound AFP-BP) during a 30 minute incubation at 4^0C. This was repeated once more. A wash with GBSS followed this before the elution of AFP by 10 mg/ml D(+)mannose diluted in GBSS in 10 mls. This step was again repeated. The column was washed with an acidic buffer solution (containing 8.76 g NaCl and 5 mls of glacial acetic acid to 1 litre ddH_2O, pH 3.0). The column was washed three times with GBSS and stored in thimersol in the fridge.

The fractions were confirmed by ELISA to contain AFP and concentrated by Centiprep 4301 (Amicon) to 1 mg/ml.

P. THE SPECIFIC INHIBITION OF MAb BINDING BY AFP

Various breast cancer cytosols (1 mg/ml) were received from Dr. William McBlain, the Hormone Receptor Laboratory. These were screened for 167H.1 reactivity by ELISA. For the binding inhibition assay, a 1/200 dilution of 9250 was done in PBS and 100 ul of this was added to each well. These plates were blocked in 2% ovalbumin-PBS. Protein inhibitor was

serially (two-fold) diluted to give a final concentration of 100 ug/ml to 3.25 ug/ml. The inhibitors were always pre-incubated for 2 hours at 37^0C before a 1/1000 dilution of the MAb (final was 1/2000) was added and incubated for 1 hour at room temperature. The plates were developed as for the usual ELISA's. Results are the means of duplicates wherein the variance was less than 5% (data not shown).

Q. PROTEIN KINASE EXPERIMENTS

Preliminary experiments had suggested that the anti-AFP-BP MAbs might have growth regulatory effects. Thus, it was important to see if the MAbs themselves may alter the phosphorylation of proteins.

Procedure of IN VIVO Kinase Experiment

1.25×10^7 G361 (human melanoma cell line) were added to small petri dishes (diameter about 45 mm) and incubated overnight in RPMI-10% FCS at 37^0C per treatment group. (These cells are adherent.) The monolayer was rinsed once with 1 ml of MEM-10% FCS (phosphate-free) then 1 ml of this media added and incubated for 1.5 hours at 37^0C to deplete the phosphate stores. 200 ul of P^{32} (0.5 mCi) was added to each plate and labeling took place at 37^0C for 1.5 hours to label intrinsic stores of ATP. The cells were then challenged with 200 ul of CH4 (1mg/ml) (constitutive) or 200 ul of pure 167H.1 (1 mg/ml) for 20 minutes. Plates were washed twice quickly with cold RPMI-10% FCS (2 mls) and 1 ml of stopping/solubilization buffer (containing fresh vanadate) was added and incubated 20 minutes on ice. 200 ul of supernatant (total) was added to 200 ul of sample buffer (see SDS-PAGE). The remainder of the supernatant was precleared thrice with 100 ul of anti-rabbit Ig sepharose and changing the tubes. 20 ul of the rabbit anti-serum was added to the precleared supernatants and incubated for 5 hours at 4^0C. 100 ul of anti-rabbit IgG Agarose (Sigma) was added to above and incubated overnight in the cold with tumbling. The agarose was washed 4 times with stopping buffer (1 ml/wash) and solubilized in 200 ul of sample buffer then loaded onto a 15% Biorad mini gel. After drying the gels were

autoradiographed overnight.

Stopping Buffer

10 mls of 1.0 M dibasic sodium phophate (pH 7.2)

9.0 g NaCl

10 mls of 100% Triton X-100

5.0 g sodium deoxycholate

1.0 g SDS

2.0 g sodium azide

0.04 g sodium flouride

Dilute to 1.0 litre with ddH_2O and adjust pH to 7.25 with 1.0 M monobasic sodium phosphate. Prior to use add vanadate to 100 uM. (Dilute 1/100 of 10 mM stock solution which is made by 18 mg/10 mls.)

III. RESULTS

A. THE 167H FUSION HYBRIDOMAS

Introduction and Scheme for the Production of MAbs

The first research objective of this project was to generate monoclonal antibodies (MAbs) to PNA reactive human tumor membrane associated glycoproteins, and/or to TF or Tn bearing glycoproteins. As it was preferable to produce MAbs to naturally occurring human tumor antigens, a source of *in situ* tumor membrane material was sought. For this, breast cancer biopsy membranes were readily available from the hormone receptor laboratory (kindly provided by Dr. William McBlain). These membranes were pooled and solubilized in NP40 as outlined in the Materials and Methods. This preparation was centrifuged at 100,000 G for one hour, the top lipid layer was discarded, and the glycoproteins in the supernatant were affinity enriched on PNA-agarose. This complex was injected into a single RBF/5Bnr mouse. No other adjuvant was used other than the PNA-agarose since Morgan et al (1984), had indicated that this adjuvant alone would effectively generate MAbs to carbohydrate epitopes.

The overall procedure for the generation of monoclonal antibodies (MAbs) to PNA reactive human breast tumor antigens is schematically depicted in Figure 1. FOX-NY was chosen as the fusion partner since it was reported that in this system there was an increased likelihood of maintaining the clone of interest. In this system the selection media prevents the expansion of hybridomas which have lost the chromosomes containing the heavy chain locus (Taggart et al, 1983). Of the 500 or so wells plated, over half of these had one or more clones present. Screening on the PNA extracts revealed the presence of high levels of antibody in essentially all wells with clones. Wells with antibody reactive to human serum albumin (HSA) were discarded. Parallel testing on ELISA plates coated with synthetic, ceramide type TF antigens (alpha and beta configurations, provided by Dr. B.M. Longenecker) were not positive for the most part (but see below). This may have been because these synthetic antigens were of a ceramide type linkage (glycolipid-like) and the immunization procedure

was designed to generate MAbs to glycoproteins. In contrast, several wells showed reactivity to the synthetic Tn antigen which was of the protein type linkage (O-serine). Well number 3A11 was one such clone which was recloned and named 167H.2 (Table 1).

Forty wells selected for their high ELISA activity to breast cancer membrane antigens, were immediately cloned. Testing of these clones revealed that less than 1% of these lost the production of antibody or had specificity for HSA (data not shown). This remarkable ability to retain the clone of interest is probably afforded by the unique property of the FOX-NY fusion system. Selection media was always used during culture of the hybridoma clones as a general precaution against loss of the clone of interest.

Selection of the 167H.1 and 167H.4 MAbs

Well number 3C10 was selected for further study as the original well showed reactivity with the synthetic TF-like (ceramide) alpha anomer (Table 1), but was no longer apparent upon recloning. It remains unknown if this change reflected batch variation of the synthetic antigens, if it was due to somatic mutation of the clone or reflected the presence of two or more clones in the original well. However, since this clone maintained strong reactions with the PNA reactive breast cancer glycoproteins, it was renamed to 167H.4 and was investigated further.

Supernatants from the 167H fusion were also screened for reactivity on frozen human tumor sections. In most cases, no antibody was demonstrated (Dr. Willans, personal communication). The apparent lack of immunoreactivity was later found to be most likely due to the use of a second antibody specific for IgG, whereas by differential testing most of the 167H fusion antibodies were found to be IgM (data not shown). It is not surprising that the short immunization protocol lead preferentially to the generation of IgM monoclonal antibodies.

Nevertheless, both the 167H.1 (3G3) and the 167H.4 (3C10) MAbs were selected for further study as they gave strong reactions in immunohistology on the majority of adenocarcinomas tested (see later). Serendipitously, both MAbs were also selected for further

study as screening of the 167H hybridoma supernatants for MAbs to the alpha-fetoprotein receptor in collaboration with Dr. Ricardo Moro, revealed that the 167H.4 MAb was able to block the binding of AFP to Ichikawa cells. The 167H.1 also shared this property. Hence, this thesis concerns the identification and characterization of the PNA-reactive antigen detected by the 167H.1 and 167H.4 MAbs.

B. 167H.1 AND 167H.4 MAbs ARE OF THE IgM ISOTYPE

Ascites were made of the 167H.1 and 167H.4 MAbs. As shown in Table 2, both ascites had reactivity with breast cancer cytosols which titrated out to more than one in one hundred thousand. Spent media of serially re-cloned 167H.1 and 167H.4 MAbs revealed both to be of the IgM isotype as shown in Table 3, using the method outlined in the Materials and Methods. This confirmed the results obtained by differential testing with anti-mouse IgG and IgM specific, second antibodies.

C. THE 167H.1 AND 167H.4 MAbs APPEAR TO RECOGNIZE CARBOHYDRATES NEAR OR AT THE AFP BINDING SITE OF THE 67 KD AFP BINDING PROTEIN

167H.1 and 167H.4 MAbs Inhibition of AFP Binding to Cells

In order to establish the authenticity of the cellular screening assay to detect MAbs specific for the AFP-BP, a comparison was made between the specific binding of ^{125}I-AFP on Ichikawa cells to PBMC's. These were established to be examples of cells which did and did not specifically bind AFP, respectively (Dr. Ricardo Moro, personal communication). That human leukemic cell lines may specifically bind AFP had been previously demonstrated (Calvo et al, 1986) as well as confirmed more recently (Lafarge-Frayssinet et al, 1989). Since it would facilitate the screening procedure by using fixed cells, a comparison was made of mild glutaraldehyde fixation to unfixed cells. As shown in Table 4, the fixation process did not seem to alter the specific binding of AFP to Ichikawa cells and did not change the expected finding that PBMC's do not specifically bind AFP beyond 5%. Thus, fixed Ichikawa cells were

used to screen the 167H fusion for MAbs which may be able to inhibit the binding of labeled AFP.

The protocol for the screening assay was specifically designed to identify only those hybridoma supernatants which contain MAbs to the AFP receptor and not those which may contain MAbs to AFP. The latter could conceivably also block the binding of AFP to cell surfaces if the hybridoma supernatants were incubated simultaneously with the labeled AFP during the binding inhibition assay. Thus, it was essential to first incubate the fixed cells overnight with the hybridoma supernatants followed by extensive washing prior to conducting the AFP binding assay. As itemized in Table 5, the 167H.1 and 167H.4 MAbs, as well as the serum of the mouse immunized for the 167H fusion (but not 30 other supernatants) were able to specifically inhibit the binding of AFP to Ichikawa cells and/or to TA3-Ha (murine, mammary adenocarcinoma cell line) cells. The inability of either MAb to completely block the binding of AFP is probably due to the inability of either MAb to react with all isoforms of the the AFP-BP (see later). As expected and as will be presented later both MAbs react with the cells used for screening. Thus, these results suggested that both the 167H.1 and 167H.4 MAbs react with a cell surface AFP receptor or with a molecule which may sterically interfere with AFP binding.

Inhibition of MAb Binding by AFP

Accumulating evidence had suggested that in addition to a cell surface AFP receptor, breast cancer tissues produce a soluble AFP binding protein (see literature survey for review). Scatchard plot analysis had suggested that the soluble AFP binding protein (AFP-BP) might be related to the cell surface receptor as its K_d was found to be intermediate of the high and low cell affinity AFP receptors of breast cancers (Sarcione et al, 1987a). As will be presented later, the 167H.1 and 167H.4 MAbs reacted with a soluble and cell surface membrane associated 67 kd antigen of breast cancer tissues. Two sources of the soluble AFP-BP were commonly employed for these investigations. A pleural effusion of a lung metastatic breast carcinoma (referred to as Ptnt 89 or PE) had been shown by ELISA and by western blotting

to contain large quantities of the immunoreactive AFP-BP (see later). Breast cancer biopsy cytosols prepared by the Hormone Receptor Laboratory were readily available for analysis (kindly provided by Dr. William McBlain).

It was rationalized that if the 167H.1 and 167H.4 MAbs could specifically block the binding of AFP to cell surfaces, then it was conceivable that AFP might block the binding of the MAbs to a soluble source of the AFP-BP, provided these MAbs reacted against the AFP binding site on the AFP-BP and did not interfere with AFP binding via other molecules.

As shown in Figures 2 to 5, the ability of AFP to inhibit the binding of the 167H.1 and 167H.4 MAbs to soluble sources of the AFP-BP were assessed. As it had already been established in the literature that human serum albumin (HSA) a molecule homologous to AFP, did not have affinity for the breast cancer cytosolic AFP binding protein (Biddle et al, 1987), this protein and an unrelated one, ovalbumin (OA) were simultaneously used to confirm the specificity of the AFP binding protein reactive with the 167H.1 and 167H.4 MAbs. In a dose related way, AFP but not HSA or OA was found to completely abrogate the binding of the 167H.1 (Figures 2, 4) and the 167H.4 (Figures 3, 5) MAbs to both PE (Figures 2, 3, results provided by Dr. Ricardo Moro), and a breast cancer cytosol, 9250 (Figures 4, 5, results performed by M.P. Laderoute). Furthermore, the one-half maximal binding inhibition concentration of AFP for the breast cancer cytosol was found to be approximately 30 ug/ml (Figures 4, 5) or 4.5×10^{-8} M. This value is coincidental with the work of Biddle et al (1987) wherein the K_d of the breast cancer cytosolic AFP binding protein was established to be 4.5×10^{-8} M. This may reflect that the two hour pre-incubation step with AFP at 37^0C prior to the addition of MAb favoured the binding equilibrium of AFP to the AFP-BP.

Several conclusions could be drawn from these sets of experiments. First and foremost, the results suggested that both the 167H.1 and 167H.4 MAbs react at or adjacent to the AFP binding site on the the AFP-BP since both MAbs specifically blocked the binding of AFP and vice-versa. Secondly, the results suggested that the cell surface AFP receptor and soluble AFP binding protein were homologous or cross-reactive entities. Thirdly, in contrast

to earlier work done in murine models (Villacampa et al, 1987), the human AFP binding protein does not appear to significantly bind HSA, which confirms the work of Biddle et al (1987).

The finding that AFP was able to completely abrogate the binding of the MAbs but neither MAb was capable of completely inhibiting the binding of AFP would be consistent with the notion that neither the 167H.1 nor 167H.4 MAb react with all AFP binding proteins but rather, may detect specific isoforms (see later) or alternatively detect separate gene-products which are homologous molecules. As will be presented later, these isoforms can be differentially expressed on certain tissues, which may also correspond to alterations in biological functions. The preliminary evidence to be presented suggests that these isoforms may involve glycosylation differences. Hence, they will be variously referred to as either glycoforms or isoforms.

The AFP-BP as a Candidate Prognostic Marker

It was already established that both the 167H.1 and 167H.4 MAbs react with 90 % of human breast cancers as assessed by the immunoperoxidase technique on frozen sections (see later). The finding that a breast cancer cytosol also displayed immunoreactive and AFP binding material, prompted the assessment of the relationship of AFP-BP levels to estrogen and progesterone receptor levels (ER/PgR status). ER/PgR values were provided by the Hormone Receptor Laboratory.

The ability to demonstrate a negative correlation of a marker to ER/PgR status is one way to demonstrate potential prognostic significance because of the following. It is believed that in breast cancers, the initial growth is under the stimulus of estrogens (Dickson et al, 1987, Frankel et al, 1987). Upon tumor progression, the cancer becomes hormone-independent associated with the loss of expression of ER/PgR and with the conversion to a more invasive, metastatic cancer. Although the factors contributing to this conversion remain to be identified, secondary to the presence of lymph node metastasis, ER and PgR have the greatest prognostic value (inclusive of Stage 0/I). Any factor which may

show a negative correlation to ER/PgR has invariably been also shown to have prognostic significance in long term clinical trials, for example the epidermal growth factor (Grimaux et al, 1989) or *c-erbB-2* (Berger et al, 1988, Slamon et al, 1987, 1989). Indeed, in the case of the latter which may be expressed in 90 % of breast cancers (King et al, 1989), transgenic models have established the involvement of this tyrosine kinase in tumor initiation/malignancy (Bouchard et al, 1989, Muller et al, 1988). Thus, it was important to address whether the AFP-BP could be found in most breast cancer cytosols and whether the levels of the AFP-BP might inversely correlate to ER/PgR.

For this the relative levels of the AFP-BP was addressed by the immunoreactivity of the 167H.1 MAb by ELISA and plotted and analysed with respect to ER/PgR levels (obtained in a blinded fashion). As shown in Figure 6, the levels of the AFP-BP showed an inverse, non-linear correlation to ER (P less than 0.001 by the Mann-Whitney U test (Sokal et al, 1981)), and was found to be less significant for PgR (P less than 0.01). In general, the ER status is a better prognostic indicator than PgR, and tumor markers which may be progression markers would be expected to show a stronger correlation to ER than to PgR. Although the results need to be extended and confirmed, and long term clinical trials are necessary to substantiate this work, it appears that the AFP-BP may be a candidate prognostic marker for breast cancers. This raises the very real possibility that the AFP-BP may be a tumor progression factor.

Molecular Evidence for a 67 Kd AFP-BP

Attempts to employ radiolabeled AFP in transblots from SDS-PAGE gels, in order to identify the molecular species of the AFP-BP in PE, did not meet with any success. As an alternative, native 5% PAGE gels (non-reducing conditions and non-denaturing conditions) were used and as shown in Figure 7, both labeled AFP and the 167H.1 and 167H.4 MAbs, detect a 67 kd band. The molecular weight was approximated using native PAGE gel molecular weight standards from Biorad, although it should be realized that the estimation of molecular weight from native gels is not as accurate as from SDS-PAGE gels under reducing

conditions as the former is less likely to resolve all complexes. However, as demonstrated in B-Lane 2, AFP did not bind to the 67 kd band in PE unless the nitrocellulose sheet had been first treated with neuraminidase (after transfer but before probing with the labeled AFP, Figure 7 B-Lane 1). The effect of neuraminidase treatment was assessed in this manner so that comparison of bands which bound ^{125}I-AFP could be directly compared to the bands reactive with anti-AFP-BP MAbs on parallel blots from the non-denaturing gels. Indeed, as shown for the 167H.1 MAb (Figure 7-A-Lanes 2 and 4) or for the 167H.4 MAb (Figure 7-A-Lanes 1 and 3), both these MAbs detect a 67 kd band by 5% PAGE whether or not the nitrocellulose sheet had been first neuraminidase treated (Lanes 3 and 4). The finding that neuraminidase enhances the *in vitro* binding of AFP, might imply that the AFP-BP contains sialic acid near the AFP binding site. Furthermore, the removal of the net negative charge of sialic acid might enhance AFP binding either by specific or non-specific mechanisms. This does not necessarily imply that AFP binding is via a carbohydrate moiety on the AFP-BP but may instead, imply that it is adjacent to the binding site. However, it has not been determined whether or not AFP binds to carbohydrates on the AFP-BP. The inability of neuraminidase to effect the binding of the 167H.1 and 167H.4 MAbs suggests that sialic acid residues, although present on the putative AFP-BP, are not involved in the binding of the 167H.1 or 167H.4 MAb.

Since the binding by AFP showed only a weak band which did not photograph very well, the blots in Figure 7 were scanned by densitometer. The band detected by AFP or MAbs to the putative receptor all coincide as shown in Figure 8. Since these blots were developed using DAB instead of the usual HRP substrate, by the time the densitometer scans were done, some of the precipitated DAB by the 167H.4 MAb was lost, so it was estimated as shown by the dotted line. Note that in the molecular weight range of these transblots which is about 10 to 500 kd, only a 67 kd band was visualized suggesting that unless a very large AFP-BP exists, there may only be one major species of an AFP-BP around a molecular weight of 67 kd, at least for breast cancer tissues.

The 167H.1 and 167H.4 MAbs Do Not Detect AFP

As mentioned earlier, it was very important to establish that the 167H.1 and 167H.4 MAbs were not reactive with AFP itself, even although the cellular binding assay was designed to exclude this possibility. A specificity for AFP if proven, could explain the ability of the 167H.1 or 167H.4 MAb to block AFP binding and conversely, the inhibition of binding of these MAbs by AFP. It was also very important to exclude reactivity of the 167H.1/167H.4 MAbs with AFP, since AFP has been reported to exhibit homotypic binding and it too has an apparent molecular weight of about 67 kd, albeit only under reducing conditions (see later).

The non-reactivity of the 167H.1 and 167H.4 MAbs with purified AFP was demonstrated in several ways. First as shown in Table 6 and representative of at least three assays, neither MAb reacts with AFP, whether hybridoma supernatants or purified MAbs were used. This was the purified AFP used for the binding inhibition experiments in Table 5 and Figures 2 and 3 and the purity is shown later. A commercial anti-serum to AFP was found to be positive when tested by ELISA showing that the purified AFP retained its antigenic nature. Thus, it is unlikely that the 167H.1 or 167H.4 MAb reacts with AFP.

Second, by Coomassie Blue staining of the purified AFP on 3-15% SDS-PAGE gels, AFP migrates with an apparent molecular weight of about 66 kd under reducing conditions, but it migrates with an apparent molecular weight of 55 kd under non-reducing conditions (Figure 9, 1 and 2, respectively). When this purified AFP was assessed by western blot with 167H.4 under non-reducing conditions (neither the 167H.1 nor 167H.4 MAb have reactivity for reduced western blots), 167H.4 did not react with AFP (Figure 10) but did react with a 62/67 kd doublet derived from the breast cancer membrane tumor extract (T) by 3-15% SDS-PAGE conditions (Figure 10) which was not detected with a control IgM MAb Y5781.4. Thus, it is not likely that either MAb to the putative AFP-BP reacts with AFP by various immunological criteria and most importantly, by molecular weight under non-reducing conditions.

The AFP Binding Site on the AFP-BP Appears to be Near or At A Potentially O-Linked Glycosylation Site

The enhancement of AFP binding *in vitro* by N'ase treatment of the western blots (Figure 7), suggested that the AFP-BP contained sialic acid and that carbohydrate determinants were near the AFP binding site. The possibility that the 167H.1 and 167H.4 MAbs detected, at least in part, carbohydrate determinants was tested by the ability of sodium borohydride ($NaBH_4$) or a much milder treatment, 10 mM periodate, to affect the subsequent binding of these MAbs by ELISA. As summarized in Table 7, it is shown that both pre-treatments of antigen significantly inhibit the binding of the 167H.1 and 167H.4 MAbs (representative of four experiments). On the other hand, the 167H.2 MAb which has proven reactivity to the Tn antigen (alpha anomer, Table 1, and Dr. B.M. Longenecker, personal communication), was not as sensitive to the periodate treatment as it was to the sodium borohydride treatment. It has been documented that mild periodate treatment does not always nor efficiently destroy all carbohydrate determinants (Feizi et al, 1987b). On the other hand sodium borohydride cleaves all O-linked carbohydrates from proteins, and this is consistent with the fact that the Tn antigen is O-glycosylated, that the 167H.2 MAb reacts with the synthetic Tn antigen, and that its reactivity was abolished by sodium borohydride. Note that since sodium borohydride is a relatively harsh treatment, comparisons were made on antigen denatured by 6M-guanidine-HCl (Table 7). The control MAbs, 7H.3 which has specificity for human DR antigens and 143H.43, specific for HSA, are not significantly inhibited by these treatments. This suggests that the 167H.1 and the 167H.4 MAbs may detect at least in part, carbohydrate determinants and it is likely that these may be O-linked to the AFP-BP.

It was already shown that neuraminidase treatment did not abrogate the binding of the 167H.1 and 167H.4 MAbs (Figure 7), but it had not been shown that the molecules detected by the 167H.1 and 167H.4 MAbs in fact bore cleavable sialic acid, only that the 67 kd molecule to which AFP bound by western blotting, likely did. In order to confirm that the putative anti-AFP-BP MAbs do detect a molecule, which like the AFP binding protein, seems to contain sialic acid, N'ase treated Ptnt 89 (also referred to as PE) was treated prior to

SDS-PAGE analysis and compared to untreated Ptnt 89 (89) by western blot analysis.

As shown in Figure 11, a two hour treatment of the antigen with neuraminidase significantly altered the migration of the bands recognized by either the 167H.1 or 167H.4 MAb (compare lane marked 89 versus the lane marked N'ase 89 for both the 167H.1 and 167H.4 MAbs). These bands were not detected by the control MAb, 7H.3 which has specificity for human Class II MHC antigens and which may detect a band at about 90 kd in the tissue extracts (non-reducing conditions), and serves as an appropriate negative control. The band reactive with the anti-AFP-BP MAbs found in Ptnt 89 migrates around 68 kd and is heterogeneous, whereas after N'ase treatment its apparent molecular weight has shifted to a lower molecular weight at about 65 kd but some heterogeneity remains. This confirms that the putative AFP-BP, as recognized by both MAbs does contain cleavable sialic acid and furthermore, along with the AFP inhibition binding studies, implies that both the 167H.1 and 167H.4 MAbs likely detect the same 67 kd molecule.

The AFP-BP was found to migrate as a 62/67 kd doublet in both benign (B) and malignant breast (T) tissues as evidenced by both the 167H.1 and 167H.4 MAbs, although the reactivity was stronger for the 67 kd band particularly in benign breast extracts (B) (Figure 11). It is not known if this might relate to the more evident unmasking of the carbohydrates (less sialic acid residues) on the AFP-BP as a result of malignant transformation or not, which might explain why there may be more of the 67 kd antigen in the benign sample (B) when compared to the relative amounts of the 62 kd band. The small differences in the pattern of the bands between the soluble secreted source of the AFP-BP (ie. 89) and membrane containing extracts (ie. B and T) suggests that there may be minor qualitative differences between secreted and membrane extracted AFP-BP. Whether this is a result of carbohydrate differences or other post-translational modifications is not yet clear. In Figure 11, the non-reactivity of the 167H.1 and 167H.4 MAb for the sodium borohydride treated antigen confirms the results obtained in Table 7. The western blot of Figure 11 was overdeveloped with the second antibody in order to enhance the photography of the blots. Thus, extra bands seen with the anti-AFP-BP MAbs are non-specific as they are found also

in the control lanes with the 7H.3 MAb. These bands were not detected in the western blots of Figures 7 and 10, which were not overdeveloped with the second antibody.

Potential Cross-Reaction of the 167H.1 MAb for TF-Like Antigens

In order to potentially elucidate the fine specificity of the carbohydrates on the AFP-BP, which appear to be recognized by the 167H.1 and 167H.4 MAbs, various naturally occurring TF antigens (the classical red cell TF antigen found on neuraminidase treated glycophorin, and a tumor associated TF antigen found on a murine mammary carcinoma mucin, epiglycanin), and synthetic TF/Tn antigens (obtained from Biomira Inc., Edmonton) were tested for 167H.1 and 167H.4 reactivity. The synthetic TF antigens tested here were different from those reported in Table 1 as they contained the glycoprotein-type linkage and not the ceramide (glycolipid-type) ones. As shown in Table 8, the 167H.1 MAb but not the 167H.4 MAb appeared to have weak but significant cross-reactivity with epiglycanin, a tumor type TF antigen. Corroborating evidence for a genuine cross-reaction of the 167H.1 MAb with TF-like determinants was the reactivity of the 167H.1 MAb for synthetic alpha or beta TF antigens which in this case, were conjugated to HSA. The non-reactivity of these MAbs for HSA was always confirmed in the recloning and cloning of the 167H.1 and 167H.4 hybridomas (as presented in Table 10 with other conjugates) Further, the ability of the PNA lectin (10 ug/ml) or epiglycanin (300 ug/ml) to block the reactivity of the 167H.1 MAb with the synthetic TF antigen implies that the 167H.1 MAb detects a carbohydrate epitope which may contain gal-galNAc or similar determinants. The inability of the 167H.1 MAb to react with the ceramide synthetic TF antigens (Table 1), indicates that the glycoprotein type linkage may partially define the 167H.1 specificity. Finally, the 167H.1 MAb does not appear to non-specifically react since it does not react with AFP (Table 6), and its reactivity for the TF antigens are significantly different from the control IgM (CH4) and the 167H.4 MAb.

Somewhat unexpectedly, the 167H.1 MAb did not cross-react with the classical red cell cryptic TF antigen found on neuraminidase treated glycophorin, although the 49H.8 MAb, which was made to and reacts with the cryptic red cell TF antigen (Longenecker et al, 1984)

clearly does under these conditions (Table 9). The specific reactivity of the 49H.8 MAb for neuraminidase-treated glycophorin was confirmed by carbohydrate inhibition. Thus, nitro-phenyl-beta-galactose but not the corresponding alpha anomer was able to almost completely block the reactivity of the 49H.8 MAb as expected (Longenecker et al, 1984). This was the first demonstration that 49H.8 reacts with the purified TF antigen (neuraminidase treated pure glycophorin), as all previous work had been performed on intact red cells. The sufficient removal of sialic acid residues on glycophorin by the neuraminidase treatment, was also shown by its PNA reactivity. Again however, the 167H.1 MAb did not display any cross-reactivity for glycophorin.

As was reviewed in the literature survey, there are antibodies which react with the tumor type TF antigens but not with those found on N'ase treated red cells. Conversely, there are also MAbs with fine specificity for the red cell cryptic TF antigen, but which do not detect tumor-derived TF antigens, such as the 49H.24 MAb (Longenecker et al, 1984). Thus, the possibility that the 167H.1 MAb might detect a tumor TF or TF-like antigen which was not significantly cross-reactive to the red cell classical TF antigen, was entertained. In this regard, it is shown in Table 9 that the 155H.7 MAb, which was made against synthetic TF antigens and which reacts with approximately 90% of common, human adenocarcinomas (Longenecker et al, 1987), also does not react with the red cell cryptic TF antigen. Although the antigen recognized by the 155H.7 MAb might be a glycolipid as ceramide type synthetic antigens were used for the immunization and screening of the 155H fusion, nevertheless it is an example of MAb which reacts to tumor TF antigens but does not cross-react with the cryptic gal-galNAc determinant of red cells.

However, the further investigation of the 167H.1 and 167H.4 MAbs on various TF/Tn synthetic antigens wherein a variety of conjugates were tested, did not prove to be meaningful (Table 10). Cross-reactions to certain batches of synthetic antigens were found to be reproducible, but the basis of these spurious reactions were not elucidated. As mentioned earlier, the 167H.1 and 167H.4 MAbs were shown not to have reactivity with irrelevant antigens such as HSA or bovine serum albumin (BSA) (Table 10). Thus, whether the weak

cross-reaction of the 167H.1 MAb for TF-like antigens is genuine or not needs to be further elucidated by the chemical analysis of the carbohydrates on the AFP-BP.

Immunoprecipitation with the 167H.1 and 167H.4 MAbs Reveals that the AFP-BP Migrates as a 67 Kd Antigen Under Both Reducing and Non-reducing Conditions

Neither MAb was reactive with protein transblots from reduced gels. Thus, in order to estimate the reduced molecular weight of the AFP-BP, the classical immunoprecipitation technique was employed. It was of additional interest to determine if other bands associating with the 67 kd AFP-BP could be specifically immunoprecipitated from ^{35}S-methionine labeled cells with the 167H.1 or 167H.4 MAb. As shown in Figure 12 for human HL-60 cells and in Figure 13 for murine P388-AD2 cells, the apparent molecular weight of the AFP-BP measured using reducing conditions was the same as calculated from non-reducing gels (ie. 67 kd). No other obvious specific bands were identified from these cells (Lane 1-167H.1, Lane 2-167H.4, Lane 3-CH4 (a control IgM). The inability to co-precipitate AFP which would have migrated at about 55 kd under non-reducing conditions was expected since these MAbs block the binding of AFP to the AFP-BP. These results confirm that the 167H.1 and 167H.4 MAbs are likely detecting the same molecule and this seems exclusive to a 67 kd moiety under reducing and non-reducing conditions, in confirmation of the earlier findings by western blots. In addition, this confirms the result of Table 5 showing that the murine equivalent of the human AFP-BP was detectable by these MAbs. Furthermore, it shows that various cell lines produce the AFP-BP and excludes the possibility that the AFP-BP was non-specifically absorbed by the FCS used for culturing purposes. Importantly, these results indicate that the AFP-BP can be distinguished from AFP in that it migrates at 67 kd under both non-reducing and reducing conditions (Figures 12, 13), while AFP migrates as a 55 kd and 66 kd band under similar conditions (Figure 9). Overall, based on several criteria, AFP can be distinguished from the putative AFP-BP, suggesting that the ability of the 167H.1 and 167H.4 MAbs to specifically inhibit and to be specifically inhibited by AFP, is due to their reactivity with AFP binding proteins.

D. DISTRIBUTION AND EXPRESSION OF THE AFP-BP

Malignant Tissues

Using the one hour incubation period for the primary antibodies, the supernatants, ascites or purified 167H.1 and 167H.4 MAbs, showed strong reactivity with common human adenocarcinomas (results supplied by Dr. Dave Willans) and with canine spontaneous tumors (results supplied by Dr. Debbie Haines) by immunohistology on frozen sections as summarized in Table 11. Approximately 90% of these tumors stained with either the 167H.1 or 167H.4 MAbs regardless of the species. For the most part, the pattern and intensity of staining was identical for both MAbs (Dr. Dave Willans, personal communication and see later). Where analysis of the heterogeneity had been documented (Tables 12, 13), 167H.1 showed minimal variability within tumor sections whereas the 167H.4 occassionally showed heterogeneity within a given tissue. These results along with previous ones, suggested that although the MAbs might be detecting the same antigen, they seem to be detecting different epitopes which might be on different isoforms of the AFP-BP (see later). This is not surprising as the 167H.1 and 167H.4 MAbs were independently generated in separate wells in the 167H fusion.

As depicted in Plate 1, these antibodies showed both cytoplasmic staining and membrane accentuation on malignant tissues which one would expect for a recirculating and cell surface expressed receptor. The expression of the 167H.1 and 167H.4 MAbs showed no gross correlation to stage of differentiation or to the metastatic status of the tumor (Plate 1). However attempts to quantitate the level of expression were not made. Indeed, unlike most antibodies to tumor associated antigens, the AFP-BP as detected by the 167H.1 or 167H.4 MAb showed little heterogeneity and for the most part, strong, uniform staining was evident. This expression would be consistent with the proposal that the AFP-BP may be an oncogene product required for malignant transformation of all cells in most adenocarcinomas. The expression of the AFP-BP was not unique to adenocarcinomas however, as some squamous carcinomas also stained (Plate 1), as did several melanomas (Table 13).

Non-malignant Tissues

Immunohistological investigations on non-malignant tissues revealed that the putative AFP-BP was not a "tumor-specific" antigen per se, as determined by either the 167H.1 or 167H.4 MAb. Glandular epithelial cells were positive, particularly the smaller ducts found in breast (Plate 2, A). However, unlike malignant tissues, membrane accentuation was not as evident. Squamous epithelium, connective tissue cells, hematopoietic cells, and most epithelial cells of major viscera were however, largely negative (Dr. Dave Willans, personal communication). Some types of benign breast were positive (proliferative and non-proliferative breast disease, Plate 2, B,C) while benign breast fibroadenomas were negative (4/4, Dr. Ricardo Moro, Dr. Dave Willians, personal communications). An example of a benign duct which did not stain with the anti-AFP-BP MAbs is given in Plate 2, D wherein the adjacent malignant breast did stain with the 167H.1 MAb. In Plate 2, E, while a hepatoma did not appreciably stain for the AFP-BP, the associated liver cirrhosis was clearly 167H.1 positive. Interestingly, other pre-malignant conditions such as for the bronchial pre-malignant mucosa in Plate 2, F, also showed expression of the AFP-BP. These results may suggest that the expression of the AFP-BP may be associated with early transformation events, as has been implied for PNA-reactive receptors (Moyer et al, 1984). However, this needs to be confirmed on a larger sample and the significance of this expression elucidated.

Since it is expected that in analogy to AFP, the AFP-BP would be an oncofetal antigen, several fetal tissues were also tested by the immunoperoxidase technique for reactivity for these MAbs. It was found that neural crest cells were positive and trophoblast cells were positive (Plate 3). The expression of the AFP-BP on trophoblast tissue is consistent with the proposal that AFP may provide an active transport mechanism to carry nutrients, growth regulators, certain fatty acids, etc., from the maternal circulation to the fetus (Mizejewski, 1985). It would be informative to more closely study the expression of the AFP-BP during fetal development to further elucidate its potential biological role.

As shown in Plate 4, for the 167H.1 or 167H.4 MAb, in normal brain, neurons and glial cells were positive for the AFP-BP.

For immunological tissues examined by the immunohistological technique, although a hyperplastic lymph node was examined (Plate 5), no evidence was obtained for T or B lymphocyte staining by either MAb. Instead, macrophages and/or dendritic cells were found to be positive. In addition, in the thymus, dendritic cell processes were clearly positive such as those associated with Hassall's Corpusles (Plate 5) whereas T thymocytes seemed to have variable weak staining which was not always easily distinguishable from stromal cell processes which may envelop developing T cells. However, as will be presented later, low density and differential expression of the 167H.1 and 167H.4 reactive antigens, were confirmed on isolated human thymocytes by FACSCAN analysis.

LoVo cells, a human colorectal cell line, was also demonstrated by the immunoperoxidase technique, to have cell surface staining as shown in Plate 6, A. A control IgM did not stain LoVo cells (Plate 6, B). In Plate 6, the human T cell leukemic cell line, Ichikawa, was cell surface labeled by 167H.4 as revealed by a rhodamine conjugated second antibody and examined by a fluorescent microscope (result kindly provided by Dr. Ricardo Moro). This was the cell line used in the cellular AFP inhibition assay for the screening of the 167H fusion for MAbs able to inhibit AFP binding (Tables 3, 4). As expected, the 167H.4 MAb was positive for a cell surface antigen on Ichikawa cells. Note that patching seems to have occurred which would be consistent with the notion that the 167H.4 MAb detects a functional cell surface receptor.

Cell Surface Staining by FACS Analysis

It had already been demonstrated that LoVo cells were positive for the expression of the antigen reactive to the 167H.1 and 167H.4 MAbs. In Figure 14, it can be seen by FACS, that 167H.1 has a higher reactivity with LoVo cells (69.73% positive, Figure 14-C) while 167H.4 detected 47.25% of LoVo cells (Figure 14-D). This broader reactivity of the 167H.1 over the 167H.4 MAb was often found (Figure 15, Table 13). The positive control for LoVo was the PNA lectin (Figure 14-A) which stained the vast majority of these cells (89.78%). The negative control used was 153H.1 (panel B), a murine MAb to the PNA lectin, used to

develop the PNA reactivity (panel A). Similarily, TA3-Ha (Figure 15), a murine, highly malignant mammary adenocarcinoma was strongly positive for the PNA lectin (94.04%, panel A), was negative for the control, anti-PNA MAb (3.82%, panel B) and showed reactivity for 167H.1 (51.71%, panel C), and for the 167H.4 MAb (21.63%, panel D). These latter results on TA3-Ha confirm the previous ones on LoVo cells regarding the higher reactivity of the 167H.1 MAb on cell lines, and suggest that the epitopes recognized by both MAbs are conserved on mouse cells, or at least on murine tumor cell lines.

A small panel of human leukemic cell lines was investigated with the 167H.4 MAb. As shown in Figure 16, HL-60 (pro-mono-myelocytic), U-937 (dendritic-like) and RAMOS (T cell leukemic) cell lines strongly expressed 167H.4 reactive epitopes, whereas MOLT-4 (T cell leukemia) did not (results supplied by Dr. Patrice Mannoni). The expression of the AFP receptor on various human leukemic cell lines was expected based on AFP binding studies (Lafarge-Frayssinet et al, 1989) as was the exception that MOLT-4 cells might not bear an AFP binding protein (Naval et al, 1985).

The lack of the AFP-BP on MOLT-4 was re-examined with both the 167H.1 and 167H.4 MAbs in parallel staining with HL-60 cells. As shown in Table 14, whereas HL-60 clearly expressed both the 167H.1 and 167H.4 reactive determinants, expression of isoforms reactive with either MAb were not found on MOLT-4 (representative of two assays). Thus, this may be taken as supportive evidence that the 167H.1 and 167H.4 MAbs likely detect commonly expressed isoforms of the cell surface AFP-BP which are notably not expressed on MOLT-4.

In sharp contrast to leukemic cell lines, fresh, human leukemic samples and the majority of cells in normal bone marrow aspirates were not found to express the AFP-BP, at least as determined by their reactivity with the 167H.4 MAb (Dr. Patrice Mannoni, personal communication). The significance of this striking difference is presently unknown but parallels the expression of PNA-receptors (see literature survey). Perhaps it may relate to the inability of leukemic cells to extravasate the circulation and/or that adaptation of blood leukemic cells to *in vitro* growth might necessitate the expression of the AFP-BP. Some

evidence supporting at least the latter possibility is provided in the section covering the biological effects of the 167H.1 MAb on HL-60 cells (see later).

Summary

Overall, the investigation of the distribution of the AFP-BP has shown that the AFP-BP is a widespread, tumor associated antigen and importantly, expressed in 90% of common, human adenocarcinomas. In addition to various types of malignant cells, these antibodies stained various fetal tissues suggesting that in analogy to AFP, the AFPr/cell surface associated AFP-BP may be another "oncofetal" antigen. The expression of the AFP-BP may be differentiation-activation associated since various non-malignant but activated cells appeared to express the AFP-BP and included: normal but involuting breast, benign and/or pre-malignant conditions, thymocytes, and dendritic/monocyte/macrophage populations. The finding that these MAbs may react with other mammalian species (canine, murine) at least suggests that *in vivo* modeling of the role of the AFP-BP would be possible in future studies.

E. PURIFICATION OF THE AFP-BP

A Two-Step Non-denaturing Purification of the AFP-BP Based on Its Affinity for AFP

Purified AFP-BP was highly desirable in order to perform further biochemical and immunochemical studies. During the purification of MAbs, it was noted that albumins are preferentially retained by the hydroxylapatite (HTP) matrix (Stanker et al, 1985). It was reasoned, since AFP and albumins are physiochemically similar (Tamaoki et al, 1983), AFP might be expected to be preferentially retained by this matrix. Furthermore, since breast cancer tissue extracts also seemed to contain a complex of the AFP-BP/AFP (Sarcione et al, 1983a, 1985, 1987a, Biddle et al, 1987, and see later), it was anticipated that the reversible interaction of AFP with its binding protein may be sufficient to cause premature elution of the AFP/AFP-BP complex at lower salt concentrations than albumin. It was similarly

expected that the association of AFP with the AFP-BP might delay the elution of the AFP-BP with other proteins which do not exhibit HTP affinity. In other words, a complex of AFP and the AFP-BP was expected to be found after the major "filtrate peak" (ie. those proteins with no affinity for HTP) and before the major albumin peak eluted with an increasing concentration of Buffer B.

This prediction turned out to be correct. As demonstrated in Figure 17, when Ptnt 89 was chromatographed on HTP, the major elution peak of the AFP-BP occurred between those with no affinity for the HTP matrix and the albumin peak, as determined by ELISA. It is noteworthy that very little protein was detected here by A_{280} in this region of the profile, suggesting most of this might be relatively enriched AFP/AFP-BP complexes. Two pools were collected. The first one corresponded to an AFP-BP late filtrate (labelled AFPr filtrate, Figure 17) whereas the second one (labelled AFPr eluate, Figure 17) referred to those fractions containing 167H.4 reactive materials obtained after the salt gradient (Buffer B) was started. Both fractions were studied further. After concentration and dialysis, the AFPr filtrate (Figure 17. Ia) or the AFPr eluate (Figure 17. IIa) was rechromatographed on a Mono Q column using the FPLC system of Pharmacia. In both cases, of the four resulting peaks, peak B gave the highest specific activity with the 167H.4 MAb as tested by ELISA (Figure 18, Ib and IIb), and the lowest reactivity with the 49H.8 control IgM MAb. Incidently, this also shows that the 49H.8 MAb does not react with peak B.

Evidence That Peak B Contains Pure AFP-BP

Further analysis of the HTP derived AFPr eluate, this time by gel filtration analysis by HPLC, showed that a major peak at 67 kd could be obtained (Figure 19. a). This 67 kd peak also showed a characteristic peak B by FPLC on the Mono-Q (Figure 19. b) and this peak was again strongly reactive with 167H.4 by ELISA (Figure 19. c). These results confirm the conclusions of western blotting and the immunoprecipitation studies that the AFP-BP has an approximate molecular weight of about 67 kd and shows that peak B contains only a 67 kd AFP-BP when purified from a soluble, secreted source of the AFP-BP (ie. Ptnt 89).

A rabbit antiserum was made to the crude HTP fraction (AFP-r Eluate) derived from Ptnt 89. This antiserum was first titrated on the crude HTP fraction to determine its activity (Table 15) which was determined to be a 1/500 dilution. Since it was suspected that the HTP-AFPr Eluate would contain AFP, the rabbit antiserum was first tested for reactivity for highly pure, commercially available human AFP. As shown in Table 16, the antiserum does not react with HSA and does react with pure AFP. This confirmed that AFP but not HSA was present in the HTP "AFPr Eluate", and indicated that this rabbit antiserum could be probed to address the purity of Peak 'B on non-reducing gels (ie. to establish that AFP was not co-purified). Western blotting with the rabbit antisera on peak B purified from PE is given in Figure 20. As shown in Lane 1 (non-reduced) only a 67 kd band was stained with the rabbit antiserum which detects AFP, AFP-BP, AFP/AFP-BP complexes and other unknown molecules. Thus, when peak B was purified from PE (Ptnt 89), it seems to have resulted in AFP-BP purified to apparent homogeneity. Note that the reactivity of the rabbit antisera is severely inhibited on reduced westerns wherein only a very weak band was noted to correspond to the AFP-BP at 67 kd (Figure 20, Lane 2) which did not photograph well. The loss of antigenic determinants on reduced westerns had been noted for both the 167H.1 and 167H.4 MAbs. Nevertheless, this confirms the previous results suggesting the AFP-BP is 67 kd under both reducing and non-reducing conditions and furthermore, suggests that the AFP-BP is likely to contain at least a single disulphide bridge as neither the 167H.1 or 167H.4 has any reactivity on reduced transblots.

However, as shown in Figure 21, when the AFP-BP was similarly purified from solubilized breast cancer membranes (biopsy samples) and not from a soluble source, a 185 kd band co-purified with peak B as evidenced by silver staining. Peak B was shown to contain mostly a 62 kd band and some 67 kd material. Since the 185 kd was only purified from membrane solubilized starting materials, it suggests that this entity may be a membrane associated antigen which might associate with the AFP-BP. This 185 kd was not found in the purified AFP-BP (peak B) as purified from PE (Ptnt 89) (Figures 19, 20 and data not shown), nor had it been specifically immunoprecipitated from ^{35}S-methionine labelled cells

(Figures 12, 13) or detected by western blots of the breast cancer membranes (T, Figure 11) with the 167H.1/167H.4 MAbs. Thus, it is not likely that the 185 kd antigen is reactive with the 167H.1 or 167H.4 MAb, but appears to associate with the 67 kd AFP binding protein. Preliminary functional evidence for a 100-200 kd antigen which may be associated with the AFP-BP in membranes will be provided later. The purified AFP-BP was sent for amino acid sequencing but apparently contained a blocked N-terminus, as a sequence was not obtained.

F. STUDIES WITH THE PURIFIED PEAK B

The AFP-BP is Specifically PNA Reactive

The PNA reactivity of the purified peak B, (AFP-BP purified from pooled breast cancer membranes) was tested by ELISA and confirmed with carbohydrate inhibition (Table 17). As a positive control antigen, the murine tumor TF antigen/mucin, epiglycanin was employed for comparison. As also shown in Table 17, PNA reactivity on both epiglycanin and the purified Peak B could be specifically inhibited by 0.1 M galactose but not appreciably by the same concentration of glucose. As well, it was confirmed that purified Peak B was both specifically 167H.1 and 167H.4 reactive as compared to the control IgM, CH4. Again, minimal cross-reactivity of the 167H.1 MAb for epiglycanin was demonstrated as was presented earlier (Table 8). The specific PNA reactivity of the 67 kd antigen was expected since the AFP-BP had been affinity enriched on PNA-agarose for the initial immunization and screening of the 167H fusion. Although PNA reactivity is preferential for gal-galNAc, it also reacts with terminal galactose residues (see later).

In corroboration of the notion that the AFP-BP contains terminal galactose residues, both IgG and IgM naturally occurring antibodies in a normal, randomly selected AB donor, could be demonstrated as reactive to the purified Peak B (Table 18). Naturally occurring antibodies could also be demonstrated to epiglycanin, a positive control TF antigen. Since the specificity of NAbs found in AB serum are usually to terminal galactose (Galili et al, 1987, 1985, 1984, Springer, 1984, Castronovo et al, 1989a,b, Gupta et al, 1983a) this might be taken

as indirect evidence suggestive of the presence of terminal galactose residues at least on some AFP-BP isoforms. This work would suggest according to the dictates of Coggin, 1986, that the AFP-BP may be a "true oncofetal" antigen, since there appears to be NAbs to this antigen. Only rarely have NAbs been demonstrated with specificity for tumor associated antigens, such as implied for the TF/Tn antigen and a **soluble** 62/67 kd PNA-reactive doublet described by Gupta et al, 1983a, as reviewed in Coggin (1986). Others have described anti-gal NAbs to a tumor associated antigen found on MCF-7 cells which appear to antagonize laminin receptors (Castronovo et al, 1989a,b), but this antigen was not characterized.

Further Evidence That The Carbohydrates on the AFP-BP Are NOT Identical to Those of Glycophorin

Although it was shown earlier that the 167H.1 MAb may recognize TF-like determinants (Table 8), and that the 167H.1 or 167H.4 MAbs might exhibit cross-reactivities to certain batches of synthetic TF or Tn antigens (Tables 8, 10), the non-reactivity of the 167H.1 MAb for desialyated glycophorin (Table 9), might reflect the fine specificity of this MAb for the AFP-BP. To further explore the possibility that carbohydrate epitopes similar or identical to the classical red cell TF antigen might be present on the AFP-BP, the rabbit antiserum to the AFP-BP/AFP which is expected to exhibit a broader specificity for the AFP-BP, was tested for reactivity to desialyated glycophorin. As shown in Table 19, the rabbit anti-serum to the AFP-BP/AFP failed to react with either glycophorin or desialyated glycophorin. That this negative reaction resulted from some unknown problem with the rabbit antibodies was ruled out by the inclusion of a positive control antigen, commercial AFP (see Table 16).

These results further substantiate the notion that the fine carbohydrate specificity on the AFP-BP is not likely identical to beta-gal-1-3-alpha-galNAc. The carbohydrates on the AFP-BP need to be elucidated by chemical analysis. Hence, it is concluded that although there may be important and unique properties shared by the AFP-BP and the elusive TF antigen of Springer (1984), the AFP-BP is not the TF antigen. However, it is not yet clear if AFP-BP

glycoforms may or may not be the Tn antigen of Springer. This is to say that the AFP-BP by virtue of the fact that it likely contains an O-glycan, must contain the core O-glycan, alpha-galNAc (Neutra et al, 1987) which is by definition, the Tn antigen. The non-reactivity of antibodies to desialyated glycophorin does not address the non-reactivity with the Tn antigen. Beta galactosidase treatment of desialyated glycophorin would be necessary to establish whether the 167H.1 or 167H.4 MAbs cross-react with the Tn antigen.

G. HUMAN AFP BEARS CRYPTIC PNA-REACTIVE SITES

It was important to examine whether AFP might be PNA reactive for several reasons. First it was essential to exclude the possibility that the AFP-BP was originally isolated on PNA-agarose for the immunization not because the AFP-BP was PNA-reactive but because AFP was PNA reactive. Secondly, conflicting reports on the prognostic significance of cryptic PNA sites in carcinomas raised the possibility that cryptic PNA reactive sites may represent discrete molecules; one of which may correlate to a good prognosis whereas the other may correlate to a poor prognosis, as might be expected for masked (terminally sialyated) AFP-BP glycoforms and for AFP, respectively.

The sole N glycan found on human AFP (Breborowicz, 1988) is identical to the N-glycan found on glycophorin (Gahmberg et al, 1988). Early work by the group of Springer et al (1976) had shown that NAbs to neuraminidase-treated glycophorin could be blocked with either gal-galNAc or gal-gluNAc moieties, which indicated that there may be NAbs to both the O-linked and N-linked carbohydrates of desialyated glycophorin. This then raised the possibility that AFP might bear cryptic PNA-reactive sites since like glycophorin, this molecule is not intrinsically PNA reactive (Breborowicz, 1988).

In order to first address this question, both the AFP purified from a hepatoma cell line (AFP^a) and used for the cellular AFP binding inhibition study of Tables 4 and 5 (Dr. Ricardo Moro), and the commercially available AFP (Dr. Wu, AFP^b) were tested for their antigenicity. This was confirmed by the use of commercially available antiserum to human AFP (Table 21). Although it was found that AFP was not intrinsically reactive with the PNA

lectin as expected (Breborowicz, 1988), upon neuraminidase-treatment, cryptic PNA reactive sites were found (Table 21). The specificity of the reaction was confirmed by inhibition with galactose (similar to epiglycanin, Table 21), but some inhibition with gluNAc was found (dissimilar to epiglycanin). The partial inhibition of PNA binding to N'ase AFP by gluNAc is not completely unexpected since a gluNAc residue is subterminal to galactose and the PNA lectin binding site seems to usually involve a disaccharide, albeit the second residue seems to play a lesser importance in its binding.

These results raise the possibility that the poorer prognosis sometimes associated with cryptic PNA reactivity of cancer tissues may relate at least in part to the expression of AFP. These results also imply that only AFP-BP glycoforms may be intrinsically PNA reactive but not isoforms of AFP. Thus, it is likely that the AFP-BP was affinity purified for the immunization for the 167H fusion by its direct interaction with the PNA lectin and NOT indirectly through interactions with AFP. This further corroborates the finding that the AFP-BP is PNA-reactive.

H. EXPRESSION OF THE AFP-BP ON CELLS OF THE IMMUNE SYSTEM

Several lines of evidence had raised the expectation that the AFP-BP should be demonstrable on cells of the immune system, particularly since AFP is known to be immunosuppressive. Direct functional testing, ie. the down-regulation of Class II MHC antigens on cells of the monocyte/macrophage/dendritic lineage (Lu et al, 1984, Crainie et al, 1989), strongly implicated that at least these cells should bear the AFP-BP. Similarly, the ability to directly grow out natural suppressor cells from the bone marrow (Hoskin et al, 1986) by culturing bone marrow cells in the presence of AFP for several weeks, might suggest that these cells also bear the AFP-BP. What was not at all clear from the literature pertaining either to the mouse or human system, was whether T cells expressed the AFP-BP. In both the murine and human systems, the group of Uriel et al had suggested that PHA activated T cells specifically bound AFP (Uriel et al, 1987, Lafarge-Frayssinet et al, 1989). However, in neither case was it shown that these were in fact T cells. Whether or not binding of AFP to

PHA activated T cells was due to a specific interaction of AFP with the type of PHA used, had not been addressed. For example, this group utilized PHA-M (mucin) which contains both PHA-L and PHA-E and it is well established that human AFP is PHA-E reactive (Taketa et al, 1989).

By immunohistology, neither the 167H.1 or 167H.4 MAb reacted with lymphocytes in an activated lymph node (Plate 5), suggesting that neither mature/peripheral T or B cells expressed the AFP-BP. On the other hand, the immunohistological study of a frozen section of human thymus suggested that some thymocytes might express the AFP-BP although it was not clear in these sections (possibly due to thickness) as to whether thymocytes express the AFP-BP or whether the staining observed may be due to the envelopment of thymocytes by AFP-BP bearing dendritic processes from stromal elements. Thus, we wished to directly assess the cell surface expression of the AFP-BP by FACS analysis of isolated human thymocytes.

The expression of the AFP-BP isoforms was addressed for unfractionated human thymocytes (derived from children usually under the age of 4), as well as on on CD45RA (p220) depleted thymocytes (predominately CD45RO+) and on CD45RO (p180+) depleted thymocytes (predominately CD45RA+). The interest in discerning whether these subpopulations of thymocytes might or might not express the AFP-BP arose from the recent proposal that those thymocytes expressing only the CD45RA (p220+) antigen (ie. those depleted for the lower molecular weight form, p180), may constitute the the generative lineage, whereas those expressing the p180 CD45RO antigen are postulated to be those committed to programmed cell death (Pilarski et al, 1989a, 1989b, Deans et al, 1989).

Weak expression of the 167H.4 antigen and moderate expression of the 167H.1 antigens could be demonstrated on unfractionated human thymocytes (Figure 22) when compared to an IgM negative control 167H.3. However, when the CD45 isoform depleted thymocyte populations were examined, reciprocal expression of the 167H.1 and 167H.4 epitopes was found. The 167H.1 MAb showed preferential expression on the CD45RA depleted subpopulation whereas 167H.4 reactivity was limited to the proposed "generative precursor population" (CD45RO-depleted) (Figure 22). Epithelial cell contamination of

either depleted population was minimal based on staining with other markers (CD2, CD45, Dr. Linda Pilarski, personal communication). The expression of the AFP-BP isoforms on the various subpopulations raises the possibility that the 167H.4 MAb may identify cells on the generative pathway, whereas the the 167H.1 MAb may distinguish those which have been committed to the death pathway. Biological evidence, corroborating this notion will be provided later.

The reported immunosuppressive effects of AFP (see literature survey) and the direct demonstration that AFP could down-regulate Class II expression on macrophages (Lu et al, 1984, 1986), and on the dendritic-like cell line P388-AD2 (Crainie et al, 1989), and earlier immunoprecipitation results (Figures 12, 13), raised the expectation that cells of the monocyte/macrophage series would express cell surface associated AFP-BP. As shown earlier, in an activated lymph node, macrophages seemed to express the AFP-BP, but not lymphocytes (T or B) (Plate 5). However, recent reports particularly by the group of Uriel et al (Lafarge-Frayssinet et al, 1989) had claimed that the expression of the AFP-BP was universal to, and induced upon PHA activation of, human PBMC or T cells. Furthermore, this group argued that the expression of the AFP receptor (AFP-BP) should to be considered as analogous to the transferrin receptor in that it performed a necessary transport system for fatty acid uptake. Since it was also shown that mRNA for AFP could be detected in PBMC, which peaked at day 3 after PHA stimulation (Lafarge-Frayssinet et al, 1990), this strengthened the argument that potentially AFP performs an autocrine function associated with T cell activation. However, if the sole function of AFP was to augment fatty acid uptake as proposed by this group, it is hard to envision why enhanced transport of an essential nutrient would lead to the inhibition of the immune system and not the reverse. For example, transferrin enhances the proliferation of immune cells. Thus, the argument that the AFP-BP should be analogous to the transferrin receptor did not at least intuitatively seem appropriate.

Many attempts to demonstrate that human peripheral T cells might express a 167H.1 reactive antigen failed, and this included a plethora of activation schemes, culturing conditions and purifications, including such as PHA, CD-3, CON A, etc. (Table 22).

It was difficult to detect any AFP-BP expression on PBMC. Variable expression of the AFP-BP was detected on larger, granular cells of stimulated or unstimulated PBMC which is thought to correspond to monocytes (Table 23). However, as shown in Table 23, a minor population of lymphocytes (usually a few percent, but occassionally higher) was found to express the AFP-BP. The number of AFP-BP positive cells was very donor dependent and it needs to be elucidated what subpopulation this might be. Studies employing AFP in the murine system imply that NK precursors might express the AFP-BP (Hooper et al, 1987). It is also known that a variable minor subpopulation of TcR gamma/delta cells exists in human blood (Hochstenbach et al, 1990) which is increased in ataxia telangiectasia (AT) individuals (Carbonari et al, 1990). Since the AT syndrome includes an immunodeficiency associated with elevated serum levels of AFP (Bridges et al, 1982), it might prove interesting to determine if AFP-BP subpopulations of lymphocytes are elevated in certain diseases or with tumor progression.

Overall, the studies on the expression of the AFP receptor on cells of the immune system confirm the finding that at least subpopulations of human monocytes/macrophages express the AFP-BP and that developing thymic T cells differentially express the AFP-BP. The AFP-BP however, does not appear to be a universal activation antigen of mature T cells, although it might demark an unknown minor subpopulation which is variably represented in the blood of some normal individuals.

I. BIOLOGICAL EFFECTS OF THE 167H.1 AND 167H.4 MAbs

Introduction

Since the 167H.1 and 167H.4 MAbs partially blocked the binding of AFP to cell surfaces, this raised the possibility that these MAbs might exhibit biological activity. A MAb which behaves as an agonist or antagonist to AFP would be very useful for determining the potential biological roles of AFP. Conversely, the ability to demonstrate that these MAbs might mimic the properties of AFP would be further evidence suggesting that these MAbs

recognize the functional, cell surface AFP receptor.

AFP has been ascribed not only immunosuppressive properties but has been shown to regulate cell differentiation and/or proliferation of normal or malignant cells as was reviewed in the literature survey. However, due to denaturation/contaminant problems with the purification of AFP, the biological effects of AFP have been contested. The results to be presented are consistent with the notion that AFP itself, mediates an inhibitory signal. Furthermore, they strongly imply that AFP, by binding to its cell surface associated binding protein, may provide the signal which subsequently generates these effects, and is not necessarily dependent upon factors which may co-purify with or bind to AFP. For these experiments unless otherwise stated, the MAbs were purified by chromatography of the respective ascites on HTP (see Materials and Methods for details). It cannot be excluded that other IgM antibodies present in ascites are not also purified. However, control MAbs purified using the same protocol should control for this possibility.

Studies on Normal Cells of the Immune System

The ability of the 167H.1/167H.4 MAb to inhibit the PHA response was investigated. After establishing the optimum concentration of PHA-P (Sigma) for mitogenesis at 2 ug/well (10 ug/ml) (Figure 23), the ability of the 167H.1 or 167H.4 ascites to inhibit the PHA induced ^{3}H-thymidine incorporation was addressed and normalized to the effect of the 167H.3 ascites. This is a negative control antibody which reacts with human PBMC (the larger, more granular subpopulation) as does the 167H.2 MAb (Table 23). Normalization to the 167H.3 ascites was necessary as ascites fluid contains undetermined levels of growth regulators/inhibitors. As shown in Figure 24, both the 167H.1 and 167H.4 MAbs showed a dose dependent inhibition of thymidine incorporation of PHA activated PBMC (normalized to the effects of the control ascites, 167H.3). Maximal suppression attained was about 63% for the 167H.1 MAb and about 40% for the 167H.4 MAb. The inability to completely suppress the PHA response was not surprising since the 167H.1 or 167H.4 MAb reacts with only a small proportion of monocytes (Table 23) which are required as antigen presenting cells for the T

cell mitogenic response to PHA. Nevertheless, the results are in accordance with the literature that AFP partially inhibits the PHA response.

Similarly, both the 167H.1 and 167H.4 MAbs were found to inhibit the one way MLR generated between two randomly selected blood group O donors (obtained from the Canadian Red Cross, Blood Transfusion Service, Edmonton). As shown in Table 24, again the suppression obtained was only partial (29 to 44%), and found to be less than the effects mediated by a crude source of active AFP (HTP isolated AFP/AFP-BP) which suppressed 51 to 75% of the response. These results are consistent with the literature and with earlier evidence showing that not all monocytes or antigen presenting cells express the AFP-BP.

The 167H.1 and/or 167H.4 MAbs were also found to block adherent cell tumoricidal activity as exemplified by chromium release (Figure 25). Importantly, it was established that these inhibitory effects, again partial, were not due to non-specific toxic effects by the MAbs. As shown in Figure 26, the viability of the adherent cell population as measured by the MTT assay, was not affected. This was also confirmed by trypan blue dye exclusion. Therefore it is considered unlikely that these partial inhibitory effects on normal cells of the immune system are due to a non-specific toxic effect of these MAbs. In fact, strong evidence supporting the contention that these purified MAbs are not cytotoxic as revealed by viability staining will be provided later.

Effects of the Anti-AFP-BP MAbs on the Proliferation of Transformed Cells

In keeping with the previously obtained results on normal cells, the 167H.1/167H.4 MAbs were similarly shown to inhibit the proliferation of P388-AD2 (a murine dendritic cell line) by as much as 62% (167H.1) or 50% (167H.4) in the MTT assay (Table 25). This was in direct contrast to IgM control antibodies, 49H.8 and CH4 which exhibited about 10% and 5% inhibition, respectively. Similar results on P388-AD2 were obtained by others (Dr. Mary Crainie, personal communication) by using thymidine incorporation.

The inhibitory effects of the 167H.1 and 167H.4 MAbs on LoVo cells (a human colorectal carcinoma) had been studied in medium containing 10% FCS by thymidine

incorporation. It was found that while inhibition with at least one of the MAbs was achieved in each assay, overall the level of inhibition varied tremendously (about 15% to 60%) for a 48 hour assay, and often both MAbs did not appear to be active at the same time (data not shown). For example, as shown in Figure 27, a dose dependent inhibition of thymidine incorporation by LoVo cells could be obtained with the 167H.1 ascites but not with the 167H.4 ascites. It is not clear whether the 167H.4 isoform was down-regulated or is more sensitive to the inhibition by AFP in the culture medium (compare Figures 2 and 3). In order to overcome these problems, a serum free medium was used (IMDM), and fortuitously, a small amount of biologically active AFP was available for the study. As can be seen in Table 26, both the 167H.1 and 167H.4 MAbs reduced the proliferation of LoVo cells as addressed by the MTT assay when compared to control values. Marginal inhibition was effected by the 49H.8 anti-TF antibody. In contrast, AFP enhanced the proliferation of LoVo cells when compared to the same concentration of HSA, an homologous protein. Furthermore, the enhancing effect by AFP could be abrogated by 50 ug/ml of either the 167H.1 or 167H.4 MAb, or by 10 ug/ml of the 167H.1 MAb but not by 10 ug/ml of the 167H.4 MAb. The relative inability of the 167H.4 MAb to abrogate the AFP mediated response is consistent with the low expression of the 167H.4 reactive isoform on LoVo cells (Figure 14). The 49H.8 MAb did not significantly affect the AFP mediated enhancement of LoVo cell proliferation. The physiological relevance of the opposing effects of AFP versus the MAbs on LoVo cells is yet to be determined, however. As was reviewed in the literature survey, growth enhancing and suppressing effects of AFP appears to relate in part to its ability to cross-link the AFP receptor since AFP aggregate formation associated with stored AFP appears to mediate effects opposite to those of deaggregated or unaggregated AFP which involves abrogation of cellular induction signals. Alternatively, AFP may transport active biological entities not found in the purified MAb preparation.

J. NOVEL IMPLICATION OF AFP AND AFP RECEPTORS IN THE ABROGATION OF CELLULAR SENESCENCE AND PROGRAMMED CELL DEATH

Apparent Growth Enhancement of HL-60 Cells By the 167H.1 MAb

Introduction

In the biological studies so far reported in this thesis, the 167H.1 and/or the 167H.4 MAbs were found to inhibit growth and/or differentiation of both normal and transformed cells. AFP, when available for parallel studies, also showed the ability to down- regulate cellular induction signals. However, the investigation of the effects of the anti-AFP-BP MAbs on the growth of HL-60 cells as measured by thymidine incorporation indicated that in 1 % Ultrasor, there was approximately a 30 % increase in thymidine incorporation associated with the 167H.1 MAb but not with the 167H.4 or control IgM MAb by 48 hours (Figure 28). This observation was reproducible and was also made by others with the 167H.1 MAb and with active AFP on HL-60 cells (tested in 2 % FCS-RPMI, Dr. Simeon Vassiliadis, personal communication). As will be developed in this section, the apparent growth enhancing effects of the 167H.1 MAb and/or AFP appears to relate to the abrogation of programmed cell death as evidenced by the promotion by these agents on the viability of HL-60 cells. Therefore, although AFP and/or AFP agonists appear to enhance the growth of HL-60 cells, this finding is in fact consistent with the previous work showing that AFP and AFP agonists down-regulate cellular induction signals. In the case of HL-60 cells, AFP and AFP agonists down-regulate the induction of programmed cell death.

The Kinetics of Enhancement by AFP or the 167H.1 MAb of Thymidine Incorporation Suggest These Agents Promote Cell Viability

An assessment of the growth promoting effects of the 167H.1 MAb and AFP was studied under stringent culturing conditions, involving the use of serum-free IMDM media. As can be seen in Table 27 (results supplied by Dr. Simeon Vassiliadis), the amount of

thymidine incorporated dwindled to almost negligible amounts by 72 to 96 hours in the media control, indicating that in this media, the cells do not survive and/or do not replicate their DNA. AFP (10 ug/ml) added at culture initiation, was able to promote the initial thymidine incorporation over the control values as measured at 48 hours (compare 9716 to 1930 cpm), but did not appreciably increase the number of cells cycling over the next 48 hours (compare 9716 to 10243 to 12214 cpm). Similarly, the 167H.1 MAb promoted a three to four fold increase in thymidine uptake of HL-60 cells as measured at the first 48 hours (compare 7590 to 1930 cpm) but the extent of thymidine incorporation did not significantly increase over the next 48 hours (compare 7590 to 7559 to 9581). Although the doubling time for HL-60 cells is unusually long, about 35 to 48 hours (Collins, 1987), these results are consistent with the notion that AFP is not a growth factor, but might promote the viability of HL-60 cells. These kinetic results were the first hint that AFP and/or AFP agonists promote the viability of HL-60 cells but do not impart a growth signal per se.

The Concept of Programmed Cell Death and Cellular Senescence

All somatic normal cells are mortal, and thus, by definition have a finite life span usually consisting of 50 (+/- 10) doublings (Hayflick, 1988), although these original studies were performed on fibroblasts, but have now been repeated on other cells. Many normal cells reach an end stage in their differentiation which involves the inability to replicate and which is known as cellular senescence. Cellular senescence can be operationally evidenced by differentiation to a non-proliferating, more mature phenotype and/or by apoptosis induction (Sugawara et al, 1990). Apoptosis, also referred to as programmed cell death (PCD) or activation induced cell death, is an active mechanism of cell death unlike necrosis. In contrast to the swelling and bursting of cells by necrosis, programmed cell death involves the induction of cell suicide machinery such as the endonuclease which ultimately cleaves the DNA into nucleosome sized pieces, and is morphologically characterized by blebbing and nuclear condensation, and by the eventual formation of apoptotic bodies (round cellular fragments) (Martin et al, 1990b). Apoptosis unlike necrosis, can be blocked by protein or mRNA

inhibitors, by growth factors and/or by other agents capable of inducing or blocking certain signals, eg. cyclosporine on developing thymocytes (Shi et al, 1989). Although the mechanisms by which specific cells in the body are eliminated are not well understood and might vary (for example aged red blood cells express a senescent antigen recognized by the immune system through which old cells are engulfed and digested; squamous cells in the skin become keratinized and are sloughed off), few cells in the body end their lives by apoptosis (Cotter er al, 1990). The use of apoptosis as a means of removing senescent cells appears to be a highly regulated and specialized event and appears to be reserved for special occassions. For example, in development of the digits of the feet and hands, programmed cell death occurs where the spaces in between the digits form. Likewise, apoptosis occurs in the development of the brain and may contribute to the apparent differences between a masculine and feminine brain (Fox, 1987). Apoptosis of developing thymocytes is believed to be necessary to eliminate self reactive cells (MacDonald et al, 1990). That bone marrow cells in general undergo apoptosis during a certain stage of their development or upon withdrawal from growth factors of long term cultured cells has only recently been appreciated (Williams et al, 1990, Martin et al, 1990b). Finally, and extremely relevant to the present investigation, HL-60 cells can be induced to differentiate to a non- proliferating state which is followed by apoptosis (Martin et al, 1990a). More recently, it has been clearly demonstrated that in cultured HL-60 cells, the "suicide machinery" may already be induced but requires an activation signal for apoptosis to occur (Martin et al, 1990b). Thus, the potential promotion of cell viability by AFP or the 167H.1 MAb as interpreted in the results in Table 27 and/or Figure 28, might relate to the abrogation of the induction of programmed cell death in HL-60 cells by AFP or AFP agonists. The condition which was common to both thymidine uptake experiments was the use of flat bottom, 96 well microtitre plates. HL-60 cells when freshly isolated from flask cultures did not show loss of viability as evidenced by trypan blue (see later). Thus, the subculture of HL-60 cells in 96 well Linbro plates was closely examined to determine whether or not HL-60 cells are given a programmed cell death signal, and what might be the source of this signal. However, before examining these experiments a discussion

of the method of viability determination is necessary.

The Use of FITC and Propidium Iodide as an Indication of Viability For Flow Cytometric Analysis

In order to easily qualify and quantitate the induction of cell death of HL-60 cells, a method of determining viability amenable to flow cytometric analysis was sought. Flow cytometric analysis was preferable, as it had been shown that dead and/or dying apoptotic cells exhibit decreased size (forward scatter, FSC) and increased granularity (side scatter, SSC) and these morphological parameters can be used for the exclusion of dead cells in regular FACSCAN analysis (Sasaki DT et al, 1987).

The dye routinely used to indicate dead cells by flow cytometric analysis has been propidium iodide (PI) which intercalates into DNA through permeable membranes of dead cells. PI is commonly employed as it correlates well to trypan blue dye exclusion viabiity staining (Sasaki DT et al, 1987), which has been and remains the standard viability determination method used in biology.

The use of propidium iodide, like trypan blue, for the study of apoptosis has severe limitations. Most notable, these dyes do not stain all apoptotic cells, nor do they demark early events in apoptosis induction such as blebbing (Martin et al, 1990b and see summary in Table 28). Shi et al (1989) used the enhanced uptake of FITC as a method to determine viability for their investigation of apoptosis induction of murine thymocytes but fluorescence was only examined by microscopy. Using the staining procedure of Shi et al (1989) for FITC uptake, and by an extensive investigation involving flow cytometric analysis and microscopy (see later), the discriminating power of FITC uptake combined with FACSCAN analysis, was verified (see Table 28). As noted in Table 28, of the five consecutive stages of apoptosis induction, trypan blue/PI stains only dead cells (non-viable apoptotic cells and cells in the process of apoptotic body formation). FITC, on the other hand, stains earlier stages and apoptotic cells. However, upon apoptotic body formation the FITC fluorochrome is lost (see later). This may be because FITC stains proteins and when the cell membranes are sufficiently

perforated, these proteins leak out. Nevertheless, by gating on forward scatter or side scatter and FITC uptake, blebbing (low FL1) can be easily distinquished from apoptotic bodies (also low FL1) (see later). Indeed, by FITC uptake, discrete stages of blebbing can be distinquished, for example at 12 hours prior to apoptotic cell formation (non-viable stage) HL-60 cells (see later).

In order to substantiate the notion that FITC may be a more sensitive indicator of cell death in HL-60 cells, a comparison of FITC to PI and in some cases to trypan blue, was investigated. As shown in Table 29, early or late passage HL-60 cells cultured for 24 hours at 1 x 10^6 per ml in flasks, or those which were purposely overgrown, were stained with both PI and FITC (see Materials and Methods for procedure) and subjected to FACSCAN analysis. Four populations were easily resolved by FACSCAN analysis and were segregated based on the relative increase in FITC or PI staining. As shown in Table 29, PI staining correlated well to the number of cells considered to be non-viable as revealed by trypan blue. However, FITC staining appeared in cells which likely were undergoing death induction but which were not yet "dead" at least as evidenced by trypan blue. That late passage HL-60 cells had a higher percentage of cells which stained with FITC but not with PI or trypan blue (compare 17.96 to 8.28 %) is consistent with this notion. Furthermore, the cultures which were overgrown (operationally defined as yellow (acidic)) showed a higher level of FITC single positive as would be expected if FITC stained dying cells as well as dead cells. PI staining, on the other hand did not appear to be as sensitive as the FITC dye. Therefore, viability determination was based on FITC uptake (and forward scatter profile). Occassionally, viable cells were defined as those cells negative for enhanced uptake of both FITC and PI.

Culturing of HL-60 Cells in 96 Well Microtitre Plates is Associated with Adherence and PCD

A notable difference between the growth of HL-60 cells cultured in flasks versus the 96 well plates is that in the former, the cells are non-adherent during their growth. In contrast, in the microtitre wells, these cells settle to the bottom within minutes, whereupon they adhere to the plastic. Adherence is operationally defined as the adhesion of the cells to

the plastic such that shaking of the plate does not jar the cells or cause them to dissociate from the surface. At 1 x 10^5 cells per well, HL-60 cells form an even monolayer across the bottom of these microtitre plates. Unlike macrophages which spread out after adhesion, HL-60 cells remain rounded. While all non-adherent cells or cell lines tested similarly adhered to the Linbro 96 well plates, adherence did not significantly occur in larger Linbro plates, in petri dishes or in flask cultures. It is conceivable that the process of adherence and/or associated homotypic binding might be associated with a cellular induction signal as it is in other systems. In the case of HL-60 cells, this might be associated with the loss of cell viability as the "suicide machinery" for apoptosis may in some cases already be induced (Martin et al, 1990b). Since the loss of cell viability appeared to be inducible (see later) and thus may relate to apoptosis induction, and since apoptosis induction is related to cellular senescence, both early and late passage HL-60 cells were investigated for their sensitivity to the induction of cell death (PCD) under various conditions in the 96 well plates. Note that HL-60 cells have been recently shown to undergo apoptosis (Martin et al, 1990a, 1990b, Cotter et al, 1990) fulfilling all the criteria of such inclusive of the ladder agarose DNA pattern.

PCD of HL-60 Cells Relates to Passage Number and Cellular Senescence

Early passage (passage number 10) or later passage (passage number 49) HL-60 cells were examined for viability after a 24 or 48 hour incubation in the 96 well plates, under optimal media conditions (10 % FCS-RPMI) or less optimal growth conditions (2 % FCS-RPMI), and at a cellular concentration where the cells form an even monolayer (1 x 10^5 per well). Viability in this case was measured by FACSCAN analysis as the percentage of cells which were double negative (no significant uptake) for propidium iodide (PI) and FITC. As can be seen in Table 30, early passage HL-60 cells did not exhibit much loss of viability when cultured in 10 % FCS-RPMI by 24 hours (about 13 %) and within another 24 hours this loss was lessened to about 6 %. This might indicate that recovery or some cell doubling occurred by 48 hours. In less enriched media (2 % FCS-RPMI), early passage HL-60 cells showed a

significant loss in cell viability (about 70 %) by 24 hours, which was reduced to 44 % by 48 hours. Again this might indicate that significant cell doubling occurred by 48 hours and suggests that even under suboptimal conditions (2 % FCS-RPMI), the amount of nutrients and/or growth factors are sufficient to support the growth of these cells, despite a death induction signal. In contrast, recovery of viability was not noted for late passage HL-60 cells at 48 hours. Furthermore, there was a loss of cell viability by 24 hours in optimal media (only 25 % viable cells) which further declined at 48 hours. In the suboptimal media, late passage HL-60 cells showed 95 % non-viable cells at 24 hours with no recovery at 48 hours. The fact that a significant loss of cell viability did not occur for early passage HL-60 cells but did for late passage, rules out non-specific cytotoxic effects of the plastic or the media and instead indicates that this death observed reflects an intrinsic property of late passage HL-60 cells. In light of the finding that these same passage 49 HL-60 cells do not undergo cell death in flasks (Table 29) but otherwise are under identical conditions, indicates clearly that the subculture of HL-60 cells on the 96 well plate is associated with an inductive signal leading to cell death. It is conceivable that the adherence and/or homotypic binding (see later) evident in the 96 well plates might be associated with the induction signal which ultimately leads to PCD.

The fact that late passage HL-60 cells which are presumably more senescent, were considerably less viable than early passage HL-60 cells, as well as the observation that this depended upon an induction signal associated with adherence in 96 well plates, provides evidence linking the death observed to PCD and to cellular senescence. The finding that an induction period of 24 to 48 hours was required and, as will be demonstrated later, death was found to be reversible, provides strong corroborating evidence that the mechanism of cell death is not necrosis but apoptosis (Martin et al, 1990b) as necrosis is lethal and thus, non-reversible (Cotter et al, 1990).

As mentioned previously, very recent studies have identified that HL-60 cells do undergo apoptosis, albeit usually after induction of differentiation with certain reagents such as DSMO (Martin et al, 1990a). It was rationalized that early passage HL-60 cells may undergo significant cell death under less stringent media conditions (eg. 5 % FCS-RPMI) but

may require a cellular differentiation signal and/or a sufficient lapse of time during which the suicide machinery might be induced. Thus, the induction of cell death of early passage HL-60 cells was studied upon 96 hours of culture at several cell densities. As mentioned above, at 1 x 10^5 cells per well, HL-60 cells form an even monolayer. At 1 x 10^4 cells per well, the cells are evenly dispersed but for the most part, do not contact each other. At 1 x 10^6 cells per well, the cells are obviously overcrowded and cells accumulated on top of the monolayer. For this analysis, a morphological interpretation of cell death by FACSCAN analysis (side scatter, forward scatter, and FITC uptake) was studied.

HL-60 Cells Appear to Undergo Apoptosis Induction in 96 Well Plates by Morphological Criteria

In Column A of Figure 29, the profiles obtained by FACSCAN analysis for 1 x 10^4 cells per well are provided. Column B gives the profiles for 1 x 10^5 cells per well, and Column C for 1 x 10^6 cells per well. The top row in Figure 29 is the morphological profile for the various cell densities where the side scatter (SSC) indicates cellular granularity and the forward scatter (FSC) indicates the size of the cells. As can be seen in this first row, as the culture density of cells increases there is a corresponding decrease in size (FSC) and the cells become more granular (increased SSC). This morphological change as revealed by FACSCAN analysis is typical of dead and/or dying cells (Sasaki DT et al, 1987) and shows that with increasing density the morphological changes in the cultured cells are consistent with significant induction of cell death. The second and third rows plot the amount of FITC fluorescence (FL1) against either forward scatter or side scatter, respectively for the different cell densities. As can be seen either by the plots with FSC or SSC, there is a corresponding increase of FITC absorbed and retained proportional to the cell density culturing conditions. The higher the cell density, the higher is the number of cells which are more strongly fluorescent. Notably, the most highly fluorescent cells were also the most granular (Column C, third row). In contrast, the smallest cell particles were the least fluorescent, possibly suggesting that upon fragmentation (or the release of apoptotic bodies), the FITC

fluorochrome is lost (Column C, second row and see later). Note the dramatic transition in FITC staining as size is diminished in Column C, Row 2. This would be consistent with an abrupt change in size upon apoptotic body release/fragmentation. A discrete subpopulation of the low density cultured HL-60 cells (Column A, Row 2) appears to be small in size but not significantly fluorescent. It may be that these cells are non-viable apoptotic cells and/or represent apoptotic bodies which are not stained by the FITC fluorochrome (see Table 28). It may be that these cells may represent a certain fraction of HL-60 cells which are known to spontaneously differentiate (Collins et al, 1987) and/or undergo apoptosis (Martin et al, 1990b). Further work will be necessary to elucidate this fine point.

By morphological and FITC dye exclusion viability staining, it can be said that a higher proportion of cells undergo cell death which correlates to higher density culturing conditions (Figure 29). As was mentioned earlier, a comparison of the results in Table 29 to Table 30 strongly implied that significant cell death was promoted in passage 49 HL-60 cells by culturing them in the 96 well plates (compare 79.18 % viable for flask cultured to 28.7 % viable in 96 well plates) under otherwise identical culturing and assay conditions. Since an obvious difference between the two culturing conditions is that HL-60 cells become adherent on the 96 well plates, it is possible that adherence may play a role in the cell death induction signal. However, this cannot be the sole induction signal since all HL-60 cells at both 1×10^4 and 10^5 per well were adherent, yet not all cells underwent cell death (Figure 29). Indeed, the first row in Figure 29 tends to support the notion that cell-cell interactions such as homotypic binding may moreso enhance the cell induction signal leading to cell death. These experiments do not quantitate the role of adherence versus homotypic binding in the death signalling.

Furthermore, at higher densities yet (Column C, Figure 29) it cannot be discerned whether these cells are dying due to nutrient/growth factor loss and/or to increased homotypic binding although both may contribute to apoptosis induction. Indeed, if the signalling pathways for these two different types of signals are different remains unknown. Recently, it has been suggested that the amount of insult/induction rather than the type of inducing agent used, determines whether the cell will die via necrosis or apoptosis; the former occurs at

higher concentrations whereas the latter occurs at lower concentrations (Cotter et al, 1990). Since even with the high density culture (Column C, Figure 29), cell death is associated with a loss of size and increased granularity which is distinguished from necrosis which involves swelling, and that cell death takes 96 hours to occur which is too long an incubation for necrosis, it is likely that in this case, death is by apoptosis.

Another point which came up as a result of the thesis defence was whether this death was activation induced cell death, programmed cell death or apoptosis. All three processes involve the same nuclear condensation, blebbing and formation of apoptotic bodies along with nuclear fragmentation and all three involve a sub-lethal hit meaning that the death induced is reversible. Dr. A.H. Wyllie who first coined the term "apoptosis" has indicated that the use of these terms reflects personal preference and not that the signalling pathway is different (Dr. A.H. Wyllie, personal communication).

To confirm that the cell death observed in Figure 29 was due to apoptosis, aliquots of the cells in Figure 29 were examined under an epi-fluorescent microscope to see if blebbing or apoptotic bodies which are typical of programmed cell death, could be recognized. Cells from Column A did not fluoresce sufficiently to be photographed. Cells from Column B, showed various stages of blebbing (photos are representative of the whole population) as shown in Figure 30 (A through D). Cells from the high density cultures (Figure 29, Column C) were found to contain cells in which the formation of apoptotic bodies was clearly evident as shown under higher magnification (Figure 30, Plate E). Thus, the morphological evidence by microscope correlates to the flow cytometer profiles and both are consistent with the premise that the mechanism of cell death is apoptosis and not necrosis, the latter which involves swelling and bursting.

In summary, the evidence provided so far suggests that by subculture of HL-60 cells in 96 well plates the cell death induced is likely apoptosis. Indeed by definition, inducible cell death implys that the mechanism is apoptosis. However, unequivocal evidence for this would be the demonstration of the endonuclease activity as revealed by the ladder formation of DNA on agarose gels. Although these experiments have not yet been performed, this evidence has

been recently provided by Martin et al (1990a, 1990b), specifically for HL-60 cells. Moreover, that the "suicide machinery" may pre-exist in cultured HL-60 cells (Martin et al, 1990b) is consistent with the work on later passage HL-60 cells in which the cells appeared to undergo apoptosis at earlier times. Finally, as will be presented later for both HL-60 cells and human thymocytes, both AFP and the 167H.1 MAb are able to reverse the events leading to cell death. This indicates that the cell death cannot be necrosis but rather must be apoptosis, as necrosis involves a lethal hit (Cotter et al, 1990).

The 167H.1 MAb Appears to Abrogate Early Signals Associated with PCD in HL-60 Cells

As was shown earlier, significant cell death can be demonstrated in early passage HL-60 cells but requires 96 hours. In Figure 31, the ability of the 167H.1 MAb to abrogate the early morphological changes associated with cell death induction was investigated since it was suspected that the 167H.1 MAb might block PCD in HL-60 cells as suggested by the results in Table 27. It was of interest to know whether this MAb abrogated the cell induction signal (an earlier event) or merely the final stages of death. Commonly, earlier stages of death which are morphologically distinguishable occur 12 hours prior to final cell death, the latter as evidenced by the uptake of trypan blue (see Table 28). Thus, as shown by FACSCAN analysis in Figure 31, at 84 hours the major portion of the cells are not smaller cells staining brightly with FITC (as was observed in Column C in Figure 29) but rather display a range of low FITC uptake, in discrete subpopulations, as shown for the control IgM (A). These discrete subpopulations may possibly represent distinct early phases of apoptosis induction such as blebbing. A similar pattern is found for the 167H.4 MAb. However, with the 167H.1 MAb, the relative level of FITC uptake is less than with either the control IgM or 167H.4 MAb. These results may suggest that the earlier events leading to cell death may be blocked by the 167H.1 MAb. However, further kinetic experiments are necessary to more precisely determine at what stage the 167H.1 MAb may block signals which ultimately result in the formation of apoptotic bodies. Further studies on the ability of the 167H.1 MAb to block PCD will be provided later. programmed cell death will be extended later.

Expression of the AFPr Negatively Correlates to Cellular Senescence

The finding that the 167H.4 MAb also detected cell surface expressed AFP-BP on HL-60 cells (Table 14, Figure 16) but appeared to lack biological effects on the enhancement of HL-60 cell growth or viability was somewhat of a paradox. In order to better understand the significance of this, the expression of the isoforms recognized by the 167H.1 and 167H.4 MAbs was studied in the context of cellular senescence. It was found that the expression of the AFP-BP as recognized by either MAb declined with cellular senescence as defined by increased passage number. As shown in Table 31, the majority of HL-60 cells at passage number 8, propagated under normal culturing conditions (condition A, flask cultured in 10 % FCS-RPMI) expressed the 167H.1 (79 %) or 167H.4 (66 %) reactive isoform. However, by passage 32, only a minority of the cells expressed the 167H.1 isoform (23 %) or the 167H.4 reactive isoform (34 %). By passage 44, the number of cells expressing the 167H.4 reactive isoform was zero. Finally, by passage 54, the AFP-BP was not detected by either the 167H.1 or 167H.4 MAb. These results suggest that the expression of the AFP-BP is inversely correlated to cellular senescence. Since the culture of HL-60 cells, in conditions where they become adherent (96 wells plates) was associated with the induction of apoptosis (see previous section), which by definition is a parameter of cellular senescence, the expression of the AFP-BP was studied on HL-60 cells subcultured in the 96 well plates. As shown for early passage cells co-induced with DMSO (Table 31) although there was some downregulation of the 167H.1 reactive isoform at 48 and 96 hours, the 167H.4 reactive isoform showed a more prominent downregulation at 48 hours (compare 35 % to 66 %) which was somewhat restored by 96 hours. DMSO is known to induce differentiation of HL-60 cells which is also associated with cellular senescence. In late passage HL-60 cells (passage number 32), almost complete downregulation of both the 167H.1 and 167H.4 reactive isoforms was found at 48 hours under condition B which that which induces PCD a facet of cellular senescence, but expression was restored completely by 96 hours. Thus, these results are additionally consistent with the notion that the expression of the AFP-BP may be inversely correlated to cellular senescence.

The 167H.1 MAb but NOT the 167H.4 or Control IgM MAb Blocks PCD of HL-60 Cells

In Table 32 the direct effects of the 167H.1 MAb on cell viability of late passage HL-60 cells was assessed. In the presence of the 167H.1 MAb, 98 % of the cells remained viable (double negative for PI/FITC) whereas in the presence of the control MAb or the 167H.4 MAb, there was a 40 % loss in cell viability. Thus, the ability of the 167H.1 MAb to promote a 30 % enhancement of thymidine incorporation as was shown in Figure 28, likely relates to the promotion of cell viability (or rescue from programmed cell death).

Further Evidence that the 167H.1 MAb May Promote Cell Viability Rather Than Impart a Bona Fide Growth Signal

As was indicated in Table 30, late passage HL-60 cells undergo a significant loss of cell viability even when cultured in 10 % FCS- RPMI in 96 well plates although this does NOT occur in suspension cultures (Table 29). FCS contains about 1 mg/ml of AFP although this is bovine and is not expected to be as active as human AFP. Nevertheless, about 100 ug/ml of bovine AFP may be present when HL-60 cells are cultured in 10 % FCS. It was of interest to know whether the 167H.1 MAb may or may not abrogate the cell death which occurred in the presence of 10 % FCS. The argument was that if the 167H.1 MAb only promoted cell viability, it would not be able to enhance the level of thymidine incorporation which occurred in the presence of 10 % FCS as this presumably contains sufficient amounts of growth factors and/or bovine AFP. Furthermore, it was rationalized that if the 167H.1 MAb only had effects on cell viability but not on growth induction, one might expect to see enhancement of thymidine incorporation with the 167H.1 MAb but not in the presence of 10 % FCS.

On the other hand, if the 167H.1 MAb was able to enhance the growth of HL-60 cells in 10 % FCS, this would support the alternative explanation that the 167H.1 MAb might provide a growth signal. The ability of the 167H.1 MAb to enhance the thymidine incorporation of HL-60 cells was tested on early and late passage numbers and in the presence of 10 % FCS or not (1 % Ultrasor) (Table 33). When HL-60 cells were cultured in 1 % Ultrasor, approximately a 20 % increase in cpm of thymidine incorporation was found at 48

hours for early passage HL-60 cells when cultured in the presence of the 167H.1 MAb. About a 30 % increase was found for late passage HL-60 cells. However, in the presence of 10 % FCS no specific enhancement of growth by the 167H.1 MAb, was found for HL-60 cells, regardless of the passage number. As expected, although the number of cells per well and the culture conditions were identical, late passage cells did not incorporate thymidine to the same level as early passage cells. This would corroborate the results in Table 30 indicating that late passage cells are more sensitive to PCD than are early passage cells. Nevertheless, the results in Table 33 might favour the argument that AFP or AFP agonists do not provide a growth signal per se, but merely promote cell viability. This interpretation is also favoured by the observation that the level of apparent growth enhancement in suboptimal media by the 167H.1 MAb, did not exceed the levels found under optimal conditions for the same passage number (compare 541,000 to 588,000 cpm for passage 10, and 363,000 to 363,000 cpm for passage 49).

AFP Blocks PCD in HL-60 Cells and Promotes Thymidine Incorporation

The ability of AFP to block PCD was investigated in parallel with thymidine incorporation on HL-60 cells (Table 34). Late passage HL- 60 cells, as expected, underwent a significant drop in cell viability by 24 hours (78 % or 88 % non-viable based on FITC or PI, respectively). In contrast, in the presence of AFP (60 ug/ml), only 31 to 33 % of the cells were non-viable by 24 hours suggesting that AFP was able to rescue a significant proportion of HL-60 cells (about 60 %) from programmed cell death. Thymidine incorporation which appears to be less sensitive an indicator (possibly because the doubling time of HL-60 cells is unusually long) showed an enhancement of thymidine incorporation of about 26 % at 48 hours. Thus, the rescue of HL-60 cells from programmed cell death by AFP seemed to correspond to a minimal increase in thymidine incorporation, evident at an early time in late passage HL-60 cells. These results are consistent with the findings in Figure 28 and Table 33 wherein 20-30 % increase in thymidine incorporation was found for the 167H.1 MAb. They are also consistent with the findings in Table 27 the latter which involved however, a less

optimal medium.

THE 167H.1 MAb BLOCKS PCD OF DEVELOPING THYMOCYTES

In order to assess the universality of the ability of AFP to abrogate programmed cell death, the effects of AFP or AFP agonists on normal cells which are known to undergo programmed cell death was investigated. To date only bona fide growth factors or hormones (Fox, 1987) have been described as inhibitors of apoptosis induction, so it was imperative to confirm the effects of AFP on PCD of normal cells, as AFP does not appear to be a bona fide growth factor. Human thymocytes are normal cells which undergo programmed cell death presumably to dispose of self-reactive clones (MacDonald et al, 1990).

Appearance of the AFP Receptor in Developing Multinegative Human Thymocytes Corresponds to Inducibility of PCD

By immunohistology there was some evidence to suggest that human thymocytes might express low levels of the AFP receptor (AFPr) (Plate 5). This was confirmed on isolated human thymocytes (Figure 22). However, FACSCAN analysis suggested that not all thymocytes express the AFPr (Figure 22). Indeed, the general lack of expression of the AFPr on peripheral lymphocytes which arise from thymic emigrants, suggested that at some phase of thymocyte development, the AFPr is no longer expressed. The possibility that the AFPr was not expressed on the earliest thymocyte progenitors was also entertained. Multinegative thymocytes, obtained by the depletion methods outlined in the Materials and Methods, were cultured in 5 % FCS-RPMI in 96 well plates and the expression of the AFP-BP isoforms followed over time. As shown in Figure 32, freshly isolated multinegative thymocytes did not express the AFPr as detected by either the 167H.1 or 167H.4 MAb (Day 0, Figure 32). This result was reproducible for three different preparations of freshly isolated multinegative thymocytes. As compared to the solid line indicating the IgM control MAb values, no expression of the AFPr was found on Day 0, 1 or 3. However, in culture these cells differentiate as evidenced by the expression of certain markers (Deans and Pilarski,

manuscript in preparation). By day 5, minor expression of the AFPr occurs with the levels of the 167H.4 reactive isoform apparently preferentially expressed over the 167H.1 reactive ones under these culturing conditions (compare 17.1 % to 24.2 % for the 167H.1 MAb and 167H.4 MAb, respectively). By day 7, there is an increase in the intensity of the expression of the 167H.1 MAb on a higher number of cells (compare 68.65 % to 29 % for the 167H.1 MAb and 167H.4 MAbs respectively). Thus, the expression of the AFPr appears upon culture during the differentiation of multinegative thymocytes.

The 167H.1 MAb but not the 167H.4 MAb Blocks PCD of Developing Human Thymocytes

Multinegative thymocytes which also adhere to the 96 well plates, undergo significant cell death on day 7 as viewed morphologically through a light microscope. In these experiments, due to the long incubation time (7 days), a slightly higher concentration of the purified MAbs (100 ug/ml) was added at culture initiation. On day 7, the cells were stained with PI/FITC in order to assess viability. As shown in Table 35, in the presence of anti-CD3 coated onto the plates, 56 % of the thymocytes were found to be non-viable (double positive for PI and FITC) whereas without anti-CD3 coated plates, a slightly lower value of 46 % non-viable cells was obtained. The ability of anti-CD3 (insoluble) to promote programmed cell death in thymocytes has been recently demonstrated by Shi et al (1989). The minimal effect found here (10 % increase) may relate to the fact that few multinegative thymocytes express high density CD3 during the first week of culture (Dr. Linda Pilarski, personal communication). The 167H.1 MAb but not the 167H.4 MAb or control IgM MAb was able to rescue almost all cells from programmed cell death as evidenced in lines 1 and 2 in Table 35 (2 % to 12 % non- viable cells). These results, demonstrating induction and reversal of PCD in developing multinegative thymocytes, is in complete agreement with the work performed on HL-60 cells, in that the 167H.1 but not the 167H.4 MAb (or control IgM MAb) blocks programmed cell death.

It was however, somewhat surprising that the 167H.4 MAb did not rescue developing human thymocytes from PCD even though this antigen was clearly demonstrable on day 7

thymocytes (Figure 32). The previous results on the expression of the 167H.1 and 167H.4 isoforms in human thymocytes (Figure 22) had suggested that thymocytes putatively committed to programmed cell death as evidenced by the exclusive expression of the CD45RO (p180) isoform, also exclusively expressed the 167H.1 reactive isoform. In contrast, thymocytes which did not express CD45RO and instead expressed CD45RA (p220), preferentially expressed the 167H.4 reactive antigen (Figure 22). The available evidence (Pilarski et al, 1989, 1990, Egerton et al, 1990) suggests that the CD45RO (p180) positive cells (which are exclusively 167H.1 reactive (Figure 22)) do not contain cells of the generative lineage whereas the CD45RA (p220) positive thymocytes do. Therefore, it is possible that the failure of the 167H.4 MAb to rescue thymocytes from programmed cell death is understandable if cells which are 167H.4 reactive do not undergo programmed cell death. It remains to be addressed whether on day 7, 167H.1 or 167H.4 positive subpopulations are mutually exclusive of each other, and whether the exclusively 167H.1 positive day 7 thymocytes are the subpopulation which undergo undergo significant cell death. For the purposes of this present investigation however, the goal was to examine the ability of the 167H.1 MAb, an AFP agonist, to abrogate programmed cell death in developing thymocytes, which was confirmed. However, from an immunological perspective, the 167H.1 and 167H.4 MAbs may provide valuable tools to elucidate the mechanisms of self- non-self recognition and/or clonal deletion of T cells in future experiments.

What is intriguing here is the notion that there may be differential regulation of the 167H.1 and 167H.4 isoforms as they relate to the susceptibility and/or programming of cell death. Although both MAbs recognize AFP binding proteins of the same molecular mass on the same tissues, they appear to recognize discrete forms of the AFP-BP which may involve different carbohydrate residues (Tables 7, 8 10) and thus are referred to as isoforms of the AFP-BP. Presently, it is not known what regulates the expression of the 167H.1 versus 167H.4 isoform, but as will be developed later, it is speculated that the preferential expression of the 167H.1 reactive isoform correlates with an inability to synthesize AFP. If this were substantiated, it would imply that the expression of the 167H.1 reactive isoform on

thymocytes committed to cell death indicates a loss of autocrine stimulation by the AFP receptor, and/or that the transcriptional blockage of the expression of AFP may be directly related to mechanisms involved in the process of programmed cell death. Finally, if the "steric hindrance mechanism of incomplete glycosylations" is true (see discussion for further details), it predicts that CD45RA subpopulations will express mRNA encoding AFP whereas CD45RO subpopulations do not. However, this hypothesis remains to be tested.

Programmed Cell Death in HL-60 Cells Revisted

The profound ability of the 167H.1 MAb to block programmed cell death of developing and probably "synchronized" multinegative thymocytes raised the possibility that if a method of synchronization of PCD for HL-60 cells could be invoked, the abrogation of cell death by the 167H.1 MAb might be more clearly apparent. As mentioned previously, apoptosis was induced in HL-60 cells with DMSO (Martin et al, 1990a). In a preliminary experiment with 1.2 % DMSO (Table 36), the percent of non-viable cells increased from 21 % at 24 hours to 31 % at 48 hours and to 47 % at 72 hours suggesting that induction of differentiation in HL-60 cells by DMSO, corresponded with induction of programmed cell death. In the presence of the 167H.1 MAb, these values were reduced and with time, a steady decline in the percentage of non-viable cells was found. These results suggest that the 167H.1 MAb may block differentiation/cellular senescence of HL-60 cells induced by DMSO such that by 48 hours significant cell division has occurred.

As a result of experiments attempting to characterize the role of CD45 in PCD, a method that appears to "synchronize" the induction of HL-60 cell death was discovered. The UCHL-1 MAb which recognizes the CD45RO (p180) isoform of CD45, was found to induce significant cell death as compared to control MAbs. As shown in Table 37, by 96 hours in 96 well plates, 26 % of mid-passage HL-60 cells were found to be viable. In contrast, treatment with the UCHL-1 MAb (30 ug/ml) was found to induce significant cell death such that only 6 % of the cells were found to be viable as demonstrated by the lack of FITC uptake. A control anti-CD45 MAb (ASH 1621) used at the same concentration as UCHL-1 did not

induce PCD and rather, may have promoted viability of the HL-60 cells. This is an important control MAb as it is the same isotype as the UCHL-1 MAb (IgG2a) and also binds to the CD45RO molecule, although through recognition of a determinant common to all CD45 molecules. The 167H.1 MAb employed at 10 ug/ml but not at 1 ug/ml, was able to block the death induced by the UCHL-1 MAb. In contrast, neither the 167H.4 or IgM control MAb used at either 10 ug/ml or 1 ug/ml was able to block the cell death mediated by the UCHL-1 MAb. These results were found in several experiments conducted under a variety of conditions. The mechanism by which the UCHL-1 MAb initiates or mediates programmed death of HL-60 cells is presently unknown. Nevertheless, for the purpose of this thesis, the ability of the 167H.1 MAb to rescue HL60 cells from programmed cell death induced by the UCHL-1 MAb suggests that in general, induction of PCD in HL-60 cells is inhibited through signals generated at the AFP receptor.

An attempt was made to optimize the conditions for the UCHL-1 induction of cell death. A higher dose of the UCHL-1 MAb (140 ug/ml) was employed on earlier passage HL-60 cells in an attempt to maximize the signal for cell death for the 167H.1 blocking studies. As shown in Figure 33, 94 % of control (media) cells cultured without the UCHL-1 MAb were found to be viable after a 96 hour culture. In contrast, in the presence of the UCHL-1 MAb, only 6 % of the cells were found to be viable. This is shown by the plot of FITC versus forward scatter, where the vast majority of cells cultured with the UCHL-1 MAb are highly fluorescent and smaller in size, typical of dead/dying cells (see Figure 29). In the presence of a control IgM MAb (100 ug/ml), the induction of cell death by the UCHL-1 MAb was not abrogated (3 % viable), whereas in the presence of the 167H.1 MAb (also at 100 ug/ml), the viability was restored to 88 % which is analogous to the control without the presence of the UCHL-1 MAb. Thus, presumably by synchronization of the induction of cell death (the UCHL-1 MAb was added to the cultures after the test MAbs had been preincubated for one hour), the 167H.1 MAb was shown to abrogate 98 % of the death induced by the UCHL-1 MAb. These results show that under optimal conditions, where induction of cell death can be maximized, shown here in Figure 33 for HL-60 and in Table 35

for multinegative thymocytes, the 167H.1 signal given at or before the PCD signal almost completely abrogates programmed cell death.

Conclusions and Summary

In summary, the paradoxical finding that AFP and AFP agonists appear to enhance the growth of HL-60 cells while inhibiting the growth of other cell types, was reconciled by the finding that these agents appear to block the induction of programmed cell death. This was shown for the HL-60 cell line, as well as for normal human thymocytes. Indeed, no evidence was found for a bona fide growth promoting effect of either AFP or the 167H.1/167H.4 MAbs on HL-60 cells other than the promotion of cell viability/abrogation of programmed cell death. These finding are in accordance with the literature which suggests that aside from the apparent growth promoting effects of AFP on natural suppressor cells, AFP is not a growth factor but rather a modulator of growth (Hoskin et al, 1985). Overall, this may be the first demonstration that AFP (as confirmed with AFP agonists) may be an anti-cellular senescence factor. It may also be the first demonstration that a natural factor which is not a bona fide growth factor, and which shows a restricted distribution of its receptor to certain developmental stages, can block programmed cell death. Indeed, that a fetal antigen like AFP may block cellular senescence is not entirely unexpected. The particular relevance of this finding to oncogenesis will be later discussed.

K. EVIDENCE THAT THE 167H.1 MAb MAY INDUCE THE PHOSPHORYLATION OF A HIGH MOLECULAR WEIGHT ANTIGEN

In an attempt to address the nature of the signalling event mediated by the 167H.1 MAb, *in vivo* (whole intact cells) protein phosphorylation experiments were conducted on G361 cells (human melanoma cells) according to the protocol outlined in the Materials and Methods.

For this experiment, after the ^{32}P prelabeled cells were challenged with either the 167H.1 or the control IgM antibody, immunoprecipitation was performed with the rabbit

antiserum which was made to the crude HTP AFP/AFP-BP fraction derived from Ptnt 89. As shown in Table 38, the 167H.1 MAb stimulated 14 fold, the specific phosphorylation of bands which could be immunoprecipitated with the anti-AFP/AFP-BP serum. As shown in Figure 34, a high molecular weight band (around 100 to 200 kd) was found to be specifically labelled with ^{32}P but only when the cells had been challenged with the 167H.1 MAb but not the control MAb. Although these results need to be extended and a proper molecular weight estimated on a different percentage gel, these results are consistent with the notion that an 185 kd phosphoprotein (see the purification of the AFP-BP from membranes (Figure 21)), may associate with the 167H.1 reactive 67 kd AFP-BP in cancer cell membranes.

IV. DISCUSSION

A. INTRODUCTION

The 167H.1 and 167H.4 MAbs Detect an AFP Receptor/Binding Protein

The observations reported in this thesis provide overwhelming evidence that a PNA-reactive, oncodevelopmental antigen is a cell surface associated and soluble AFP binding protein recognized by the 167H.1 and 167H.4 MAbs. The major lines of evidence supporting this notion are the finding that both MAbs specifically block the binding of AFP to cell surfaces (Table 5), AFP specifically blocks the binding of these MAbs to soluble sources of the AFP-BP (Figures 2 - 5), and that the AFP-BP was purified on the basis that it binds reversibly to AFP (Figures 17, 18, Table 16). Furthermore, this 67 kd protein was easily distinguished from AFP on immunological (Tables 6, 17, 21, Figure 10) and biochemical criteria (Figures 9, 11, 12, 13), indicating that it is a discrete entity. The finding that the 167H.1 and 167H.4 MAbs appear to be AFP agonists (Tables 24 - 27, 32 - 37, Figures 24, 25, 27, 28, 31, 33) can be taken as further evidence that these MAbs recognize a functional component of the AFP receptor. Indeed, these AFP agonistic MAbs may be very useful to further elucidate the functional role of AFP in oncogenesis, differentiation, and development, since it has proven to be difficult to purify active AFP for these studies.

The AFP-BP/Receptor Has An Approximate Molecular Weight of 67 Kilodaltons

Gel filtration analysis indicated that a 67 kd moiety reacted with the 167H.4 MAb (Figure 19). This entity (peak B) was further determined to be reactive with the 167H.1 MAb (Table 17). Further analysis either by immunoprecipitation (Figures 12, 13) or by western blotting (Figure 10, 11) also identified the AFP-BP recognized by both MAbs to be a 67 kd moiety by SDS-PAGE analysis. Finally by native PAGE gels (Figure 7), both the MAbs and radiolabelled AFP identified a 67 kd antigen by western blotting in corroborating evidence of the above biochemical findings. These results imply that in the molecular weight range of

approximately 10 to 500 kd, only a single species of the AFP-BP/receptor exists, at least for the tissues examined. However, it cannot be presently excluded that a high molecular weight AFP-BP exists or whether or not there is more than one gene which encodes an AFP-BP.

B. THE NATURE OF THE CELL SURFACE FUNCTIONAL AFP RECEPTOR

Gross biochemical or immunological differences between the soluble and cell surface associated AFP-BP were not established (Figure 11). Attempts to sequence the AFP-BP at the protein or mRNA level were not met with success. It remains to be determined whether or not the cell surface associated AFP-BP contains a transmembrane segment. Nevertheless, preliminary evidence suggests that a 185 kd protein may associate with the cell surface associated AFP-BP (Figure 21) and may become phosphorylated upon activation of the AFP receptor (Figure 34). The identity of this protein and further characterization of the functional AFP receptor needs to be elucidated.

C. THE NATURE OF THE 167H.1 AND 167H.4 REACTIVE ISOFORMS

Preliminary evidence suggests that both the 167H.1 and 167H.4 MAbs appear to detect at least in part, carbohydrate determinants on the AFP-BP. This was evidenced by the sensitivity of the reactions of the MAbs to periodate and to sodium borohydride (Table 7). However, with the more recent advent of high quality commercially available glycanases, these results suggesting that an O-glycan is involved need to be confirmed with the appropriate enzymes. That there may be some cross-reactivity of the 167H.1 MAb to TF-like determinants (Table 8, 10) provides further evidence that it may detect carbohydrate structures which contain terminal galactose. That the glycoforms recognized by either MAb may involve distinct entities is substantiated by the following observations. In immunohistology, certain cancers appeared to express only the 167H.4 reactive isoform such as a transitional cell carcinoma, a renal cell carcinoma (Table 12) and a poorly differentiated squamous cell lung carcinoma (Table 13). Late passage or induced HL-60 cells appeared to preferentially lose the expression of the 167H.4 reactive antigen as demonstrated directly by

FACSCAN analysis (Table 31) and/or as was inferred by the inability of the 167H.4 MAb to mediate biological effects (Tables 33, 34, 38 and Figures 28, 31, 33). The expression of the 167H.1 or 167H.4 MAb reactive antigens appear to be developmentally and differentially regulated on human thymocyte subpopulations (Figure 22) or expressed on functionally different thymocyte subpopulations (Table 35). That there may be several isoforms of the AFP-BP can be interpreted from the work of Hosokawa et al, 1989. Using an unique MAb to AFP which was unusual in that it reacted with cell surface associated AFP on some but not all hepatomas, they showed by two dimensional electrophoresis that about 11 spots migrating between 62 and 69 kd with corresponding pI's of about 5.6 to 6.6 could be visualized in addition to bands which correspond to AFP (pI's of 4.8 and 5.2). Thus, it is proposed that the 167H.1 and 167H.4 MAbs, due to their monoclonal nature, may detect a discrete subpopulation of the AFPr/AFP-BP isoforms which are differentially regulated with differentiation and/or during development in both malignant and normal tissues.

D. THE STERIC HINDRANCE MECHANISM OF INCOMPLETE GLYCOSYLATIONS: AN HYPOTHESIS

The work performed on purified AFP-BP (peak B) suggests that the AFP-BP may be PNA-reactive (Table 17). This was expected since the 167H fusion was made against PNA-reactive glycoproteins isolated from breast cancer membranes (Figure 1). Additionally, since there may be naturally occurring antibodies (NAbs) of both the IgG and IgM isotypes to this antigen (Table 18), this may suggest that the AFP-BP may be analogous to the PNA-reactive, TF antigen of Springer (1984) which is thought to undergo incomplete glycosylations associated with tumor progression of cancers. Other supporting evidence for the similarity between these two antigens are that both are expressed in 90 % of adenocarcinomas, and may contain O-linked glycans (Table 7). Indeed the AFP-BP may contain a TF-like hapten (Tables 8, 10), but which is thought not to be identical to the beta-gal-1-3-galNAc hapten as represented on desialyated glycophorin (Tables 8, 9, 19).

It is proposed that the AFP-BP may undergo incomplete glycosylations associated with the tumor progression of cancers, as has been proposed for PNA-reactive antigens (Springer, 1984, Reading et al, 1985). Furthermore, it is proposed based on circumstantial evidence that the 167H.1 reactive glycoform may be a more mature glycosylation variant than the one(s) recognized by the 167H.4 MAb. Observations consistent with this hypothesis include the following. The expression of the 167H.4 reactive isoform appears on potentially earlier, less committed human thymocytes as judged by the co-expression of the CD45RA (p220 +) isoform (Figure 22) and may likewise be analogous to anaplastic tumors which may preferentially express the Tn antigen which is the precursor of the TF antigen (Springer, 1984, Springer et al, 1989, 1986, 1985a). The 167H.1 MAb might recognize TF-like antigens, and shows a higher expression (Figures 14, 15) and less heterogeneity (Tables 12, 13) than the 167H.4 MAb which would make the 167H.1 reactive isoform more analogous to the TF antigen. The loss of expression of the 167H.4 antigen may be more sensitive to cellular senescence inducing schemes (Table 31) suggesting not only that its expression may be more tightly regulated but that it may be inversely correlated to the differentiation state. This again appears to be similar to the Tn antigen (Springer et al, 1985a, 1989). The rare finding that tumors may only express the 167H.4 reactive antigen parallels that found for the Tn antigen (Springer et al, 1989, 1986, 1985a). However, it remains to be conclusively shown by chemical analysis that the carbohydrate(s) recognized by the 167H.1 MAb may be more mature than the one(s) recognized by the 167H.4 MAb.

As was reviewed in the literature survey, the hypotheses which have been put forth to explain the unmasking phenomenon of PNA-reactive glycoproteins associated with tumor progression (Springer 1984, Leathem et al, 1987) have not been adequate. This is because they fail to describe how the specific unmasking of a PNA-reactive glycoprotein defined by naturally occurring antibodies may occur in the presence of the fully functional, often overactive, relevant glycosyltransferases. With these important considerations in mind, the steric hindrance mechanism of incomplete glycosylation was formulated.

As a fundamental aspect of this hypothesis, it is proposed that AFP may form a complex with the AFP-BP prior to the completion of post-translational modifications (glycosylations) of the soluble AFP-BP. Some evidence for this already exists in the literature (Sarcione et al, 1985). Accordingly, the more AFP co-produced within a cell relative to the amounts of AFP-BP, the more immature (qualitatively and quantitively) the glycoforms of the AFP-BP produced. The more AFP produced might correspond to a more malignant phenotype of the tumor (see later discussions) which is expected to correspond to tumor progression. Thus, the expression of the 167H.4 reactive epitope may be an indicator of the relative levels of AFP co-produced which may be connected to increased malignant potential.

Important observations are consistent with the notion that the relative levels of AFP produced compared to the AFP-BP, are heightened with tumor progression. Morimoto et al (1988), have shown that with tumor progression associated with overt and/or distant metastasis, grossly elevated levels of free AFP are found in the tumor and in the serum, despite the inability to demonstrate the presence of free AFP at earlier stages by immunological means. At earlier stages, it is proposed that the co-expression of the AFP-BP may antigenically mask the presence of AFP (Sarcione et al, 1987a). This relative increase in immunologically detectable AFP is common to most cancer cell types with tumor progression (reviewed in Morimoto et al, 1988), suggesting that the overproduction of AFP may contribute to malignant behaviour in general for solid tumors.

Since the expression of AFP is tightly regulated at the transcriptional level (Tamaoki et al, 1984), this implies at least that the expression of the 167H.4 reactive glycoform may also be more stringently regulated compared to the 167H.1 reactive isoform, if the steric hindrance hypothesis is correct.

Clearly, the steric hindrance mechanism of incomplete glycosylations needs to be addressed by examining the expression of the putative, AFP-BP glycoforms in respect to the expression of mRNA for AFP. As well, it remains to be conclusively shown that the 167H.4 reactive glycoform is a precursor to the 167H.1 reactive one. Nevertheless, this hypothesis is invaluable as it can easily be tested and may be the first to account for the correlation of the

specific unmasking of a PNA-reactive glycoprotein to tumor progression, common in adenocarcinomas.

E. POTENTIAL BIOLOGICAL ROLE OF AFP AND AFP RECEPTORS

The biological investigations suggested that for the most part, the 167H.1 and 167H.4 MAbs behave as AFP agonists. The results reported here are entirely consistent with the prevailing notion that AFP or AFP agonists abrogate cellular induction signals involved in proliferation (Tables 25, 26, Figure 27) and/or differentiation (Table 24, Figures 24, 25, 27) of normal or transformed cells. Of prime relevance to oncogenesis, these investigations with AFP agonists have corroborated that AFP, by binding to its receptor, may generate immunosuppressive signals in the appropriate responding cells such as monocytes/macrophages.

The ability of the MAbs to mimic the effects of AFP was an important finding. As extensively reviewed in the literature survey, there have been significant problems with the purification of active AFP. Indeed, since AFP seems exquisitely sensitive to denaturation and often highly pure AFP is not biologically active, this has led to the speculation that either a contaminating substance contains the biological activity ascribed to AFP or that AFP does not contain any biological activity. It is unlikely that the purified MAbs would contain substances which would co-purify with AFP. Thus, the finding that these MAbs exhibit AFP agonistic activity provides compelling evidence corroborating that AFP by binding to its receptor mediates a biological signal. Furthermore, that the 167H.4 MAb lacked biological activity under circumstances in which the 167H.1 MAb exhibited activity (Figures 28, 31, 33, Tables 27, 32, 33, 35, 37), strongly argues that the purified MAbs do not contain other substances other than the monoclonal antibodies which have biological activity.

On the other hand one might argue that the AFP preparations might contain other unknown biologically active substances even although they were not revealed by SDS-PAGE (Figure 9). For the purposes of this thesis, due to the general unavailability of active AFP, most of the biological studies were performed with the MAbs to the AFPr and not with AFP.

However, in more recent experiments, preliminary evidence does suggest that dehydroepiandrosterone is a newly described AFP antagonist and this steroid precursor but not its sulphated derivative the latter which is known not to contain any *in vitro* biological activity, specifically blocks the ability of AFP (and not the 167H.1 MAb) to rescue MN thymocytes from PCD in a dose responsive fashion (data not shown). This would argue that it is AFP which abrogates programmed cell death.

In addition, as expected from the literature (Lester et al, 1977), we found that only active AFP could block the binding of the MAbs to the soluble AFP-BP, as the commercially available AFP (purchased from Dr. Wu) was not biologically active (Figure 17) and did not compete for binding to the AFP-BP (data not shown). It is tempting to speculate that in general active AFP would be already complexed with its soluble binding protein meaning that the active species of AFP would not be available for purification by affinity chromatography with antibodies to AFP. Thus, through this reasoning it may not be so surprising that the AFP commonly purified has no activity. In this regard the newly described purification procedure for AFP (see Materials and Methods, Section O) should be useful for future investigations into the biological roles of AFP. Future work to confirm the biological activities of AFP should not only involve genetic manipulations but the use of AFP antagonists (e.g. dehydroepiandrosterone) would serve to verify this point.

Interestingly, the biological results were extended to the abrogation of cellular senescence in transformed cells (HL-60, Tables 27, 32, 34, 37, 38 and Figures 28, 31, 33) and in normal cells (developing thymocytes, Table 35). Cellular senescence can be operationally evidenced by differentiation to a non-proliferating more mature phenotype and/or by apoptosis induction (Sugawara et al, 1990). Although growth factors often block cellular senescence as evidenced on the ablation of apoptosis, a more precise definition of an "anti-cellular senescence factor" may be a factor without demonstrated growth potentiating activities but which may nevertheless block apoptosis and cellular senescence. The finding that AFP and AFP agonists inhibit proliferation (and thus are not growth factors) (Tables 24-26, Figures 24, 27), but do block apoptosis (Tables 27, 32, 33-37, Figures 28, 31, 33), suggests

that AFP may be more appropriately considered an "anti-cellular senescence factor" than bona fide growth factors. The inverse expression of the AFPr with cellular senescence (Table 31) which is remarkably absent at passage 54, strongly supports the contention that the expression of the AFPr may block cellular senescence, perhaps by an autocrine mechanism. The loss of the AFPr by passage 54 is also in keeping with the Hayflick number which was established to be a maximal of 50 +/- 10 cell doublings before normal cells are unable to reproduce themselves (reviewed in Hayflick, 1988). Finally, since the expression of the AFP-BP appears to be more restricted, at least on hematopoietic cells, than those for established growth factors, IL-2, IL-3, and CSF-1 which may also abrogate apoptosis (Nieto et al, 1989, Williams et al, 1990, Martin et al, 1990), this would imply that AFP and AFP receptors (and/or associated molecules) appear to play an specialized role in the abrogation of cellular senescence and/or regulation of programmed cell death, moreso than typical growth factors or hormones. On the basis of the above, it is proposed that AFP be considered an "anti-cellular senescence factor".

The finding that AFP may appear to enhance the growth of cells by its ability to abrogate cellular senescence suggests that the uncommonly reported growth enhancing effects of AFP may relate to this phenomenon. For example, AFP has been reported to behave as a growth factor for developing murine bone marrow, natural suppressor (NS) cells (Hoskin et al, 1985), which is entirely consistent and/or analogous to the work on HL-60 cells. Pro-monomyelocytic cells such as HL-60 cells are considered to be one subtype of NS cells (Noga et al, 1988). As well, there is accumulating evidence that bone marrow cells undergo apoptosis induction after prolonged culture and withdrawal of growth factors (Williams et al, 1990). Finally, and just recently, that HL-60 cells undergo apoptosis upon differentiation (Martin et al, 1990a) and in some cases, cultured HL-60 cells may already have the "suicide machinery" induced (Martin et al, 1990b, Cotter et al, 1990), are in keeping with the findings reported here for HL-60 cells. Thus, the ability of AFP to behave as a growth factor in unusual situations such as on certain bone marrow cells, seems to correspond to the findings that these unusual situations involve programmed cell death. On the other hand, whether the

rarely found growth promoting effects of aggregated AFP on estrogen responsive tissues (Mizejewski et al, 1987) relate to the abrogation of cellular senescence or some other mechanism, needs to be carefully addressed.

It is possible that AFP or AFP agonists may have similar effects on other cells which are known to undergo programmed cell death, but which are not transformed, such as developing thymocytes (MacDonald et al, 1990). The evidence suggests that AFP and AFP agonists may block cell death of developing multinegative (CD3,4,8-) human thymocytes (Table 35) and that the phases of thymocyte development associated with the inducibility of cell death (ie. by day 4 to 5 of cultured multinegative thymocytes) are associated with the co-expression of the AFP receptor (Figure 32). That is to say that since significant cell death occurred on day 7, the induction signals for apoptosis must have occurred earlier. If the results in Tables 30 and 31 for HL-60 cells can be extrapolated to human thymocytes, then the signal may have occurred at about 48 hours earlier. In contrast, most CD3 positive cells in peripheral blood whether activated or not do not express the AFP-BP (Tables 22, 23) indicating the expression of the AFP-BP is largely restricted to the development of thymocytes (Figures 22, 32, Plate 5). Whether peripheral NK cells, TcR gamma-delta cells or NS cells usually express the AFP-BP as do monocytes (Table 23, Plate 5), however, remains to be explored.

In fractionated human thymocyte subpopulations, it was found that the 167H.1 glycoform of the AFP-BP is preferentially expressed on the CD45RO (p180+) subpopulation whereas the 167H.4 glycoform is exclusively expressed on the CD45RA (p220+) subpopulation (Figure 22). This differential expression of the AFP-BP glycoforms is intriguing in light of recent proposals that the CD45RA isoform may demark the generative lineage of developing thymocytes whereas the CD45RO isoform may indicate those already committed to cell death (Pilarski et al, 1989a, 1989b, Egerton et al, 1990, Deans et al, 1990, Deans, 1990). Although at the present time it is not known what mechanism controls the differential expression of the 167H.1 versus 167H.4 reactive glycoforms, it was hypothesized earlier that the 167H.1 reactive cells do not co-produce AFP whereas the 167H.4 reactive cells

do. Thus, the commitment of thymocytes to the death pathway is postulated to be associated with the loss of expression of AFP, the exclusive appearance of the 167H.1 isoform and the conversion of cell surface CD45RA to CD45RO, the latter requiring a few days (Deans, 1990). Northern blotting for mRNA for AFP on CD45 isoform subpopulations should discern this point in future experiments. Nevertheless it is speculated that the autocrine stimulation of the AFP receptor (ie. cells which produce AFP, the AFP-BP and/or ancillary proteins) may abrogate cellular senescence and/or programmed cell death of normal cells.

Others have previously speculated that AFP may be involved in the regulation of programmed cell death of normal cells (Fox, 1987) although the results presented here may be the first direct demonstration to this effect. However, confirmation of apoptosis induction and rescue by AFP and AFP agonists will require the demonstration of endonuclease activity as evidenced by a ladder pattern of the DNA on agarose gels.

Although it is not well accepted that most cancer cell types produce AFP since it cannot easily be antigenically detected until late in oncogenesis (Morimoto et al, 1988), the work provided by the group of Sarcione et al (Sarcione et al, 1983a, 1985, 1987a, 1987b, Biddle et al, 1987) has suggested that this may be because the soluble AFP binding protein, which has been characterized with the 167H.1 and 167H.4 MAbs, masks the presence of AFP by complex formation. Hence, after mild denaturation, AFP is readily demonstrated in most if not all breast cancer cytosols and/or serum. Indeed, the AFP-BP has been purified from breast cancer tissues by its affinity for AFP, the latter which has affinity for hydroxyapatite (Figures 17 - 19, Table 16). The widespread expression of the AFP-BP, for example in 90 % of common, human adenocarcinomas may be the first inclination that AFP may be ubiquitously expressed in human oncogenesis, but has been masked by the soluble AFP-BP. This however remains to be addressed by *in situ* hybridization for mRNA encoding AFP.

What role might AFP and AFP receptors play in human oncogenesis? From the results reported here it is suggested that the autocrine stimulation of the AFP receptor may contribute to the long term intrinsic growth potential (immortality) of tumors by blocking cellular senescence. Since it has been strongly interred that AFP **itself** (and not other

contaminating factors) imparts a down-regulatory and/or immunosuppressive signal as evidenced by the agonistic activities of the 167H.1 and 167H.4 MAbs, the proposals of others that AFP secreted by tumors may abrogate host immunological defence mechanisms (Sarcione et al, 1985, Mizejewski, 1985), has been re-affirmed. The secretion of an immunosuppressive molecule is expected to greatly enhance the uncontrolled growth and metastasis of the tumor. Furthermore, AFP may have untoward effects on developing immunocytes which may also contribute to the immunopathology associated with late stages of cancer such as the atrophy of the thymus (Tanaka et al, 1987). Finally, and very importantly, since cell mediated tumoricidal activity involves programmed cell death of the tumor (Ucker, 1987), it is additionally proposed that the autocrine stimulation of the AFP receptor may be associated with intrinsic resistance of malignant tumors to certain forms of host cell mediated cytolysis (Cotter et al, 1990). Hence, AFP antagonists may serve to counteract several aspects of the malignant behaviour of tumor cells and thus may well prove to be useful for the clinical therapy of human cancers.

Overall, through various autocrine and paracrine mechanisms, this work suggests the expression of AFP and its receptor may be intimately related to the malignant growth potential of human tumors. Indeed, a simple method to directly test this possibility is by the development and use of AFP antagonists to abrogate oncogenesis, since in rodent models (Kamata, 1985) and in human hepatoma (Kusumoto et al, 1983), there has been some encouraging evidence of this.

F. CLINICAL RELEVANCE OF AFP AND AFP BINDING PROTEINS TO HUMAN BREAST CANCER

The finding that the AFP-BP is strongly expressed in 90 % of human breast cancers as evidenced by immunohistology on frozen sections (Table 11, Plate 1), and that AFP can be found in most if not all breast cancer cytosols (Sarcione et al, 1987b, 1983a) or elevated in breast cancer serum (Sarcione et al, 1987a), raises the possibility that AFP and AFP binding proteins might play an important role in breast cancer oncogenesis. The results presented in

Figure 6 suggest that the levels of the soluble, cytosolic AFP-BP as recognized by the 167H.1 MAb may be upregulated with tumor progression and may closely correlate with the conversion of breast cancers to hormone independence. As such, the AFP-BP may be a novel, candidate prognostic marker for breast cancers but which clearly needs to be addressed in long term studies.

A comparison of the relationship of the AFP-BP to ER/PgR status, to the other established prognostic markers, reveals that it is unique. The AFP-BP is similar to *c-erbB-2* in that it is expressed in the vast majority of breast cancers (90 %, King et al, 1989, Wright et al, 1989), but unlike *c-erbB-2* where a negative correlation to ER/PgR often is not established or is weak (Tandon et al, 1989, Slamon et al, 1989, 1987, Berger et al, 1988), shows a highly significant negative correlation (P less than 0.001, Figure 6). On the other hand, the non-linear, inverse correlation of the AFP-BP to ER/PgR is analogous to the *c-erbB-1* oncogene (Pekonen et al, 1988, Bauknecht et al, 1989, Grimaux et al, 1989), but differs in that *c-erbB-1* is only expressed in about 33 % of breast cancers (presumably, expressed in breast cancers which have already converted to hormone independence). This, then raises the possibility that the AFP-BP may be a more useful marker than the *c-erbB* oncogene family, to address the current hormone status associated with tumor progression of breast cancers. It is also noteworthy, that the correlation of the AFP-BP levels to ER level was more significant than that established to PgR levels. This would be expected for a tumor progression marker as the ER has higher prognostic significance than PgR. Future work should elucidate whether AFP-BP levels, particularily in concert with the *c-erbB-2* and *c-erbB-1* markers, may provide prognostic significance at or beyond the levels demonstrated for lymph node status.

Others have attempted to establish a correlation of the levels of cytosolic AFP-BP to ER/PgR status (Biddle et al, 1987). In this case, and in contrast to the findings reported here, a positive correlation was inferred. Presumably, methodological differences would account for the discordant results as this group indirectly assessed the levels of the AFP binding protein based upon the binding of radiolabeled AFP.

The potential of the AFP-BP as a prognostic marker for breast cancers also raised the possibility that it may be useful for a serum screening assay for early breast cancer detection. However, studies of fifty or more cancer sera did not reveal significant differences in the amount of AFP-BP detected by either the 167H.1 or 167H.4 MAb when compared to normal control sera (Dr. B.M. Longenecker, personal communication). The source of the soluble AFP-BP detected in normal blood needs to be elucidated, but monocytes in peripheral blood express the AFP-BP (Table 22, 23). Thus, the AFP-BP is unlikely to provide the basis of an early cancer serum screening assay.

Other Lines of Evidence Implicating AFP and/or AFP Receptors in Breast Oncogenesis

Other lines of indirect evidence may implicate AFP specifically in human breast cancer. Ataxia telangiectasia (AT) is a genetically inherited, autosomal recessive disorder marked by thymic atrophy, overt immunodeficiency, and the overexpression of AFP as detected in the serum (Bridges et al, 1982). Whereas breast cancer is not commonly found in homozygotes as they may not survive to maturity, breast cancer is commonly affiliated with female heterozygotes which carry one defective AT allele. At least 20 % of breast cancer cases are estimated to contain a defective AT allele (Swift et al, 1986, Gatti et al, 1988). The actual incidence of AT mutant alleles involved in human breast cancer awaits the identification and localization of all complementation groups which may be greater than four. The most commonly occurring AT allele has however been mapped to 11q22-23 (Gatti et al, 1988). Whether a candidate, affected gene is the *ets-1* oncogene as proposed by Laderoute (1990) or not (Concannon et al, 1990), or maps to a related oncogene, *tpl-1* (Bear et al, 1989), remains to be established. Interestingly, the *tpl-1* gene appears to encode a tumor progression factor which is homologous and closely linked to the *ets-1* oncogene.

Nevertheless, the disease ataxia telangiectasia provides an interesting aberration linking AFP overexpression to thymic atrophy, immunosuppression, and developmental disorders. The expression of the AFP-BP on developing thymocytes (Figures 22, 32), on PBMC macrophages/monocytes (Tables 22, 23) and in the fetus (Plate 3) further corroborates this

notion. Furthermore, thymic cortical atrophy is not only noted in the AT syndrome, but is associated with mammary carcinomas (Fu et al, 1989), and with other carcinomas (Tanaka et al, 1987). Future studies aimed at the elucidation of the AT mutant alleles should provide a better understanding of the role of AFP in T cell development and in breast cancer oncogenesis. That there may be connectivity between these two processes potentially involving recombination/repair machinery, has been speculated (Laderoute, 1990). Indeed recent evidence showing that the *scid* mutation in mice, known to involve an Ig/TcR rearrangement defect, also involves an ionizing radiation repair defect in non-lymphoid cells (Fulop et al, 1990) is in keeping with the aforementioned notion. Whether this connectivity also involves estrogen stimulated growth needs also to be investigated as there is some evidence that estrogens may stimulate hemopoiesis (Hayama et al, 1983) or induce serum AFP levels in adults in both males and females (Hau et al, 1984).

It remains to be determined by molecular approaches, whether AFP might be a prognostic indicator for breast cancers. For example, in teratomas, the prognostic significance of AFP has been established in a small sample (Malogolowkin et al, 1989). On the other hand, as the results shown in Figure 6 imply that the AFP-BP may be upregulated with the progression of breast cancers, it is conceivable that, in analogy to laminin receptors (Castronovo et al, 1990), the upregulation of the AFP-BP may concur with the upregulation of AFP and/or vice-versa. Furthermore, accumulating evidence suggests that AFP is upregulated with tumor progression of most solid tumors (Morimoto et al, 1988). Thus, it is anticipated that both the AFP-BP and AFP might be shown to be upregulated with tumor progression in breast cancers. However, this remains to be determined.

Overall, the available evidence raises the notion that AFP and the AFP-BP may play a potentially causative role in tumor progression of breast cancers. Whether the putative 185 kd phosphoprotein which may associate with the AFP/AFP-BP complex is related to the **orphaned** *c-erbB-2* tyrosine kinase which is also 185 kd in size, may be expressed in 90 % of breast cancers (King et al, 1989), and relates to tumor progression of breast cancers, will be very important to resolve.

G. SUMMARY

The working hypothesis that there may be a single, PNA-reactive glycoprotein expressed in 90 % of common, human adenocarcinomas to which there may also be naturally occurring antibodies, was tested. The approach taken, namely the generation of MAbs to PNA-reactive breast cancer biopsy materials, successfully resulted in the identification of two monoclonal antibodies, 167H.1 and 167H.4, to a 67 kd, PNA-reactive antigen expressed in 90% of adenocarcinomas, and both IgG and IgM naturally occurring antibodies could be demonstrated to the purified antigen.

The function of this antigen in oncogenesis appears to relate to its role as an alpha-fetoprotein receptor/binding protein. Although the precise biological autocrine function of the AFP-BP in oncogenesis needs to be fully elucidated, it appears that this component of the AFPr may mediate the effects rendered by AFP binding. This appears to be the abrogation of cellular induction signals relating to cell proliferation and/or differentiation. In preliminary experiments, these effects were extended to the inhibition of programmed cell death. This may implicate AFP and AFP binding proteins in the abrogation of cellular senescence and thus might play an important role in the immortality of tumors. Since cellular immune mediated lysis of tumor targets involves programmed cell death (Ucker, 1987), an autocrine function of AFP may also relate to the resistance of malignant tumors to some immune mediators. The paracrine function of the 67 kd AFP-BP which may be released from the tumor as a complex with AFP, likely relates to the immunosuppression of the host and/or blockage or disregulated development of T cells. That AFP is itself an immunosuppressive molecule was corroborated by the 167H.1 and 167H.4 MAbs which appear to be AFP agonists. Hence, the corollary of the working hypothesis, that the PNA-reactive antigen may play an important role in oncogenesis which also may relate to the immunosuppression of the host, was substantiated. The potential importance of the PNA-reactive antigen to breast oncogenesis can also be inferred by the ability to establish it as a candidate prognostic indicator.

Significantly as a result of this investigation, the steric hindrance mechanism of incomplete glycosylations was formulated to explain how glycosylation changes in a PNA-reactive antigen may relate to tumor progression. In this hypothesis it was proposed that AFP may form a complex with the AFP-BP and sterically hinder the continuation of post-translational glycosylations. This is expected to result in incomplete glycosylations of the AFP-BP, proportional to the amount of AFP produced, the latter which may correlate to tumor progression.

Finally, this work would be consistent with the notion that the AFP-BP is a PNA-reactive, tumor progression factor, the latter which was the initial premise of this work.

Overall, this work implies that AFP and AFP binding proteins and/or receptors may play important roles in human oncogenesis and consequently, may pave the way for the development of novel diagnostic, prognostic and therapeutic modalities, at least for breast cancers. The clinical use of AFP antagonists should ultimately prove or disprove the hypothesis that AFP and/or AFP receptors may be major, malignancy conferring oncogenes for human solid tumors.

Table 1. Reactions of the 167H.1, 167H.4 and 167H.2 MAbs With Synthetic Tn and TF Antigens

	O.D. 405 nm		
	TFα	TFβ	Tn
PNA (1/100)	.834	1.164	.000
PNA (1/300)	.430	1.220	.006
Anti-PNA Control	.301	.011	.000
Media Control	.240	.000	.000
49H.8	1.109	1.172	1.318
49H.24	.313	.073	.000
167H.2E10	.269	.021	.535
167H.3F5	.326	.000	.757
167H.1H1	1.051	.000	1.394
167H.3A11 (167H.2)	.301	.002	1.198
167H.3C10 (167H.4)	.800	.000	.000
167H.3G3 (167H.1)	.269	.000	.000

ELISA results are the means of duplicates. The plates were blocked in 5% FCS-PBS. All MAbs are culture supernatants except the anti-PNA Control was the 153H.1 ascites used at 1/1000. The stock solution of PNA (Peanut Agglutinin lectin, EY Labs) was 1 mg/ml. The code names for the synthetic antigens were as follows: CTV=TFαHSA, T1-B6=TFβBSA, PTC=TnαHSA.

The 49H.8 and 49H.24 are IgM MAbs to tumor and/or red cell cryptic TF antigens (Longenecker et al, 1982, 1984). Both the TFα and TFβ antigens were of the ceramide type linkage whereas the Tnα was a glycoprotein type linkage. The results indicate that the 167H.2 MAb has reactivity for the synthetic Tn antigen and that the original supernatant of the 167H.4 MAb contained some reactivity for an αTF hapten. Most of the supernatants in the 167H fusion lacked reactivity with these synthetic carbohydrate antigens.

Table 2. Titration of 167H.1 and 167H.4 Ascites on a Breast Cancer Cytosol by ELISA.

	O.D. 405 nm		
Dilution of MAb	**167H.1**	**167H.4**	**Control**
1/10	**/** [a]	1.969/1.928	.538/.499
1/100	1.944/1.864	1.764/1.831	.108/.142
1/1000	1.811/1.627	1.337/1.421	.047/.056
1/10,000	1.316/1.370	.378/.355	.046/.061
1/100,000	.335/.303	.081/.096	.061/.095

The background was 0.056/.044 and the control was an ascites with no MAb present. The values given are readings on duplicate wells.

a) The values exceeded the reader limits of 2.0.

Table 3. Isotyping of the 167H.1 and 167H.4 MAbs by ELISA.

Anti-murine Antibody Isotype	167H.1	167H.4	Media	193H Fusion Serum
IgG1	.030	.034	.031	.599
IgG2a	.028	.032	.030	.855
IgG2b	.019	.027	.024	>2.000
IgG3	.026	.024	.030	.176
IgM	.292	.327	.022	.874
IgA	.027	.036	.024	.771

Isotyping was performed in duplicate, as outlined in Materials and Methods, on hybridoma supernatant collected in serum-free media. Results are expressed as the means of duplicates and provided by Mary-Jane Meeker. The results indicate that both the 167H.1 and 167H.4 MAbs are of the IgM isotype. The positive control used was the serum of a mouse immunized for the 193H fusion.

Table 4. Effect of Fixation of Cells on the Specific Binding of AFP

	^{125}I-AFP BINDING (cpm)		%
	Without COLD AFP	With COLD AFP	Competition
Non-Fixed Ichikawa	6502 (462)	2654 (903)	59 %
Non-Fixed PBMC	1843 (259)	1757 (228)	5 %
Fixed Ichikawa	2404 (272)	583 (76)	76 %
Fixed PBMC	3914 (919)	3712 (185)	5 %

Results are expressed as the means and standard errors ( ) of quadruplicates and are provided by Dr. Ricardo Moro. The assay was conducted at 4°C according to the methodology outlined in the Materials and Methods. The cold (unlabeled) AFP used for inhibition was a 200 fold excess. The results indicate that mild gluteraldehyde fixation does not alter the specific binding of AFP to Ichikawa cells and the lack of binding to normal, unactivated PBMC.

Table 5. Inhibition of AFP Binding to Cells by the 167H Fusion MAbs or Serum

ICHIKAWA CELLS:	^{125}I-AFP Binding (cpm)	% Competition
Control (no cold AFP)	5799±1559	-
Control (plus cold AFP)	638± 48	100%
167H Fusion Serum (1/300)	2159	71%
167H.4 R1 Supernatant	2040	73%
167H.2B6 R2 Supernatant	5839	0%
167H.2D7 R3 Supernatant	6152	0%
30 Other 167H Hybridomas Reclone Supernatants		0%
TA3-Ha CELLS:		
Control (no cold AFP)	10721± 623	-
Control (plus cold AFP)	5181± 239	100%
167H.4 R1 Ascites (1/200)	6512± 150	76%
167H.1 R1 Ascites (1/200)	6355± 257	79%
Control NMS (1/100)	11070±1211	0%

Results are the means and standard errors of quadruplicates except the screening of the 167H fusion supernatants on Ichikawa cells which were performed in duplicate (wherein only the means are provided). Results are provided by Dr. Ricardo Moro. The supernatants or antibodies were first incubated overnight at 4°C on the fixed cells before washing three times, and then the binding assay was conducted as outlined in the Materials and Methods. The preincubation step was necessary to avoid the detection of MAbs to AFP rather than the AFPr. Cold AFP refers to a 200 fold excess. The specific activity of the labeled AFP was 8×10^6 cpm/ug of AFP. NMS refers to normal mouse serum. The TA3-Ha cell line is a murine, mammary adenocarcinoma. The 167H.2B6 and 167H.2D7 MAbs are examples of reclone supernatants containing high levels of antibody reactive to breast tumor membrane antigens but which do not block the binding of AFP.

Table 6. Neither the 167H.1 or 167H.4 MAb React with AFP by ELISA

	O.D. 405 nm		
MAb	AFP	Glycophorin	N'ase Glycophorin
167H.1 sup	.142 (.019)	.148 (.013)	.179 (.018)
167H.4 sup	.168 (.031)	.147 (.007)	.170 (.016)
CH5 sup	.158 (.019)	.146 (.010)	.167 (.015)
167H.1 (1/100)	.220 (.022)	.153 (.009)	.188 (.018)
167H.4 (1/100)	.224 (.017)	.157 (.007)	.207 (.019)
49H.8 (1/100)	N/A	.154 (.004)	.977 (.036)
CH4 (1/100)	.193 (.022)	.149 (.005)	.191 (.010)
Anti-AFP (1/500)[a]	2.112 (.321)*		
NRS (1/500)[b]	.134 (.004)		

All antigens were coated at 1 ug/well. Hybridoma supernatants (sup) were used neat. Purified IgM MAbs (1 mg/ml) were diluted 1/100 in 1% BSA-PBS. CH4 and CH5 are IgM control MAbs. Results are the means of quadruplicates and standard errors ( ) are shown. Pure human AFP (see Figure 9) was the preparation used for the binding inhibition assay, the glycophorin was purchased from Sigma. N'ase (neuraminidase) treatment is outlined in the Materials and Methods and was performed after blocking the plates with 1% BSA-PBS (KPL). The results indicate neither the purified 167H.1/167H.4 MAbs (from ascites) or hybridoma supernatants have affinity for AFP.

N/A = not applicable

a) Commercially available rabbit antiserum to human AFP (Chemicon).
b) NRS = normal rabbit serum.

*$p<.0005$ when compared to NRS.

Table 7. The Binding Site of the 167H.1 and 167H.4 MAbs Appears to Involve Carbohydrates and May Involve O-Glycans

	O.D. 405 nm (% INHIBITION)					
	PERIODATE [a]			6M GUANIDINE-HCL [b]		
EXPERIMENT I:	(-)	(10 mM)		(-)	($NaBH_4$)	
167H.1	1.161±.299	.333±.055	99%	1.018±.124	.530±.102	87%
167H.2	.724±.169	.533±.055	34%	.580±.061	.365±.097	100%
167H.4	1.305±.143	.101±.026	100%	1.075±.074	.518±.011	89%
7H.3	1.150±.099	.793±.150	36%	.930±.041	.718±.038	56%
143H.43	1.627±.183	1.571±.123	4%	1.362±.170	1.135±.054	43%
Control Y5781.4	.128±.014	.139±.030	-	.084±.052	.412±.026	-
EXPERIMENT II:						
167H.1	.537±.092	.155±.022	93%	.848±.078	.159±.016	88%
167H.2	ND	ND	ND	.645±.077	.081±.009	98%
167H.4	.489±.130	.086±.010	100%	.786±.080	.118±.012	93%
Control Y5781.4	.064±.009	.122±.047	-	.105±.019	.069±.011	-
7H.3	ND	ND	ND	.453±.028	.420±.059	14%
143H.43	.567±.093	.623±.090	0%	ND	ND	ND
193H.31	.522±.086	.330±.034	55%	1.368±.037	.046±.016	100%
155H.7	ND	ND	ND	.815±.041	.171±.016	87%
Control 153H.1	ND	ND	ND	.037±.004	.064±.000	-

...continued on next page.

Table 7. (continued)

Results are the means and standard errors of quadruplicates by ELISA. % inhibition was calculated after subtracting the control MAb values. 7H.3 is a MAb to Class II (common) determinants. 143H.43 is a MAb to HSA. 193H.31 is a MAb produced to PNA reactive glycoproteins. 155H.7 is MAb to synthetic TF antigens.

a) After Ptnt 89 was added to plates and before blocking, half the plate was treated with 10mM periodate or buffers alone as outlined in the Materials and Methods.

b) Since sodium borohydride ($NaBH_4$) is a harsh treatment and is thought to denature glycoproteins, comparisons of the effects of this cleavage method were assessed on 6M guanidine-HCl denatured Ptnt 89 (see Materials and Methods).

ND = Not Done.

Results demonstrate that the epitopes recognized by the 167H.1 and 167H.4 MAbs are sensitive to mild periodate or sodium borohydride pretreatment whereas other MAbs to protein determinants (7H3, 143H.43) are not. Sodium borohydride specifically cleaves O-glycans and not N-glycans.

Table 8. The 167H.1 But Not 167H.4 MAb Reactivity Appears to Cross-React with the gal-GalNAc TF Hapten as Tested by ELISA

	O.D. 405 nm		
	TF (alpha)	TF (beta)	Epiglycanin
167H.1	.331 (.081)**	.364 (.093)*	.189 (.035)*
167H.4	.144 (.010)	.132 (.022)	.087 (.014)
CH4	.101 (.024)	.091 (.023)	.042 (.008)
167H.1 / 30 ug Epi	.159 (.030)		
167H.1 / 1 ug PNA	.106 (.012)		

The synthetic TF antigens used in this study were the glycoprotein type conjugated to HSA. A comparison was made to N'ase glycophorin (the classical RBC TF antigen) and to another tumor TF antigen, epiglycanin which is of murine origin. For the inhibition of 167H.1 binding to the TF alpha anomer, either 30 ug of epiglycanin (Epi) or 1 ug of PNA per well was employed. Purified MAbs (1 mg/ml) were used at 1/100.

*$p<.005$
**$p<.01$

Table 9. Neither the 167H.1 or the 155H.7 MAb React with the Cryptic Red Cell "Classical" TF Antigen

	O.D. 405 nm	
	Glycophorin	Neuraminidase Glycophorin
167H.1	.110 (.010)	.163 (.023)
167H.4	.031 (.002)	.030 (.001)
49H.8	.050 (.003)	1.238 (.037)
49H.8 + Nitro-phenyl- -Gal	.048 (.002)	.095 (.006)
49H.8 + Nitro-phenyl- -Gal	.046 (.003)	1.132 (.007)
155H.7	.075 (.005)	.100 (.007)
CH4 IgM Control	.025 (.003)	.035 (.004)
PNA[b]	.066 (.008)	1.268 (.013)

These ELISA results were performed under the same conditions as for Table 6 using purified MAbs. All MAbs are IgM except 155H.7. The PNA used here is a biotinylated one which was then developed using the Avidin-HRP conjugate as outlined in the Materials and Methods.

Table 10. The Anti-AFP-BP MAbs Can Show Cross-Reactions to Certain Batches of Synthetic Tn or TF Antigens

	O.D. 405 nm		
Experiment I:	167H.1	167H.4	Control IgM
Ptnt 89	.926	.575	.304
Tn-HSA-2	1.405	.634	.490
Tn-HSA-RK033.11	.186	.103	.200
Tn-HSA-RK033.12	.164	.177	.179
Tn-HSA-RK033.13	.158	.117	.153
Tnβ-HSA-RK033.6	.170	.120	.162
HSA	.184	.109	.186
AH188.07(TFαHSA)	.406	.280	.208
Experiment II:			
Ptnt 89	.969	.697	.116
KLH	.256	.257	.165
HSA	.145	.081	.109
AH188.14(TFαKLH)	.193	.233	.116
82-90P(TFαKLH)	.202	.109	.090
RK191-01(TFαHSA)	.124	.070	.082
AH140.41(TFαBSA)	.236	.325	.101
BSA	.112	.256	.089

ELISA results are the means of duplicates and results were supplied by Diane Swanlund. Unless marked otherwise, the synthetic antigens are of the alpha galNAc O-linkage. The ascites were used at 1/300.

Table 11. Summary of 167H.1 and 167H.4 MAb Reactivities on Adenocarcinomas by Immunohistology on Frozen Sections by the Immunoperoxidase Method

	#POSITIVE/#TESTED (% POSITIVE)	
	167H.1 MAb	**167H.4 MAb**
CANINE SPONTANEOUS TUMORS:		
Breast	7/7 (100%)	7/7 (100%)
Colon	5/5 (100%)	4/4 (100%)
Lung	4/5 (80%)	4/5 (80%)
HUMAN ADENOCARCINOMAS:		
Breast	28/30 (93%)	14/16 (88%)
Colon	14/14 (100%)	10/10 (100%)
Lung	20/26 (77%)	4/4 (100%)

Frozen sections of cancer tissues were stained by the ABC immunoperoxidase method (see Materials and Methods). Results were provided by Dr. Dave Willans (human) and Dr. Debbie Haines (canine).

Table 12. Incidence of Heterogeneity of the Antigens Recognized by the 167H.1 and 167H.4 MAbs by Immunohistology

	MAb	# Positive/ # Tested	Intensity and Number			Incidence of Heterogeneity # Positive
			++	+	-	
Colon Carcinoma	167H.1	9/9	5	4	0	1/9
	167H.4	4/4	1	3	0	0/4
Breast Carcinoma	167H.1	9/10	6	3	1	1/9
	167H.4	6/6	5	1	0	1/6
Lung Carcinoma	167H.1	7/10	3	4	3	0/7
	167H.4	4/4	2	2	0	2/4
Summary:						
	167H.1	25/29 (86%)				2/25 (8%)
	167H.4	14/14 (100%)				2/14 (14%)
Transitional Cell Carcinoma of Urinary Bladder	167H.1	0/1	0	0	0	N/A
	167H.4	1/1	1	0	0	0/1
Renal Cell Carcinoma	167H.1	0/1	0	0	0	N/A
	167H.4	1/1	0	1	0	1/1

Legend as per Table 11.

N/A = Not Applicable.

Table 13. Heterogeneity and Relative Intensity of Staining of the Anti-AFP-BP MAbs in Immunohistology

Cancer Type:	MAb	Relative Intensity	Heterogeneity
Colonic Adenocarcinoma (X91)	167H.1	+	-
	167H.4	++	-
	155H.7	++	-
Leiomyosarcoma, Intermediate Grade (X222)	167H.1	+	-
	167H.4	+	-
	155H.7	+	-
Squamous Cell, Lung Carcinoma (X288)	167H.1	++	-
	167H.4	++	++
	155H.7	++	++
Colonic Adenocarcinoma (X861)	167H.1	++	-
	167H.4	++	-
	155H.7	++	-
Colonic Adenocarcinoma Grade IV (X367)	167H.1	++	+
	167H.4	++	+
	155H.7	++	+
Poorly Differentiated Squamous Cell Lung Carcinoma (X378)	167H.1	-	N/A
	167H.4	+	++
	155H.7	+	++
Metastatic Embryonal Carcinoma of Testes (X416)	167H.1	++	-
	167H.4	++	-
	155H.7	++	+
Metastatic, Small Cell, Undifferentiated Carcinoma (P126)	167H.1	+	-
Metastatic Breast Cancer (X16)	167H.1	++	-
Metastatic Melanoma (Spindle Cell Type) (X135)	167H.1	+	+
Prostatic Adenocarcinoma (X154)	167H.1	++	-
Renal Cell Carcinoma (X71)	167H.1	-	N/A

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Table 13. (continued)

Cancer Type:	MAb	Relative Intensity	Heterogeneity
Medullary Carcinoma of Breast (X338)	167H.4	+	+
Well Differentiated Lung Adenocarcinoma (X406)	167H.4	+	-
Renal Cell Carcinoma (X407)	167H.4	+	++
Adenocarcinoma Metastatic to Brain (Primary Unknown) (X424)	167H.4	++	-
Poorly Differentiated Gastric (X216)	167H.4	-	N/A

Legend as per Table 11.

Codes: Intensity: ++ very strong, + strong-medium, - negative.
Heterogeneity: ++ much, + some-little, - none.

N/A = Not Applicable.

(X---) = Frozen section identification number.
(P---) = Paraffin section identification number.

Table 14. Molt-4 Does Not Express the AFP-BP as Determined by the 167H.1 or 167H.4 MAbs by FACSCAN Analysis.

	% Positive [a]	
	Molt-4	HL-60
167H.1	0%	23%
167H.2	99%	34%
167H.3	0%	34%
167H.4	0%	17%
7H.3	0%	0%

a) % positive by FACSCAN analysis after subtraction of relevant isotyped matched control antibody.

Table 15. Titration of Rabbit Antisera Made to the Crude AFP/AFP-BP Derived from HTP Column

Reciprocal of Dilutions:	ELISA O.D. 590 nm	
	Pre-Serum	30 Day Post
100	.072	1.936
200	.062	1.788
400	.056	1.526
800	.052	1.045
1600	.066	.629
3200	.047	.263
6400	.050	.128
12,800	.046	.085
25,600	.027	.049
51,200	.048	.053

Results are expressed as the mean of duplicates of ELISA results on the crude AFP/AFP-BP complex isolated by HTP chromatography and was developed with alkaline phosphatase labeled goat anti-rabbit (Biorad) used at 1/750. By the third immunization (30 day post) the antiserum displayed reactivity out to greater than a 1/10,000 dilution. The rabbit had been immunized thrice, every 10 days.

Table 16. The Demonstration that the Rabbit Antisera Made to Crude AFP/AFP-BP (HTP Fraction) Reacts with AFP but not HSA

	ELISA O.D. 590 nm	
	AFP	HSA
PNA (1/200), Anti-PNA (1/500)	0.052 (.002)	.054 (.007)
Anti-PNA (1/500)	0.043 (.001)	.043 (.006)
Anti-AFP[a] (1/500)	1.657 (.188)	.133 (.009)
Anti-AFP/AFPr (1/500)	1.105 (.064)	.120 (.004)
Normal Rabbit Serum	0.048 (.002)	.047 (.005)

Results are expressed as the means and standard errors of triplicates asessed by ELISA. AFP was commercially purchased (Wu, 1984), and pure HSA was purchased from Sigma. Development was as per Table 15, for the rabbit antisera (Anti-AFP, Anti-AFP/AFP-BP, and the normal rabbit serum). The PNA (1 mg/ml) reactivity was developed with the 153H.1 murine MAb to the PNA lectin diluted 1/500.

a) The anti-AFP antiserum was a commercially available one (Chemicon).

Table 17. Purified AFP-BP (Peak B) is Specifically PNA Reactive by ELISA

		O.D. 405 nm		
	Inhibitor	Peak B	Epiglycanin	HSA
PNA, 153H.1	-	.647 (.006)	.673 (.030)	.054 (.007)
153H.1 (Control)	-	.162 (.014)	.043 (.009)	.043 (.006)
PNA, 153H.1	D-gal	.204 (.016)	.292 (.030)	
PNA, 153H.1	D-glu	.533 (.032)	.702 (.014)	
167H.1	-	.481 (.033)	.195 (.035)	
167H.4	-	.421 (.025)	.063 (.009)	
CH4	-	.059 (.010)	.016 (.000)	

Approximately 1 ug/well of purified Peak B was coated overnight onto Nuclon plates in PBS and processed for ELISA as described in the Materials and Methods. The PNA lectin was used at 500 ng/well in 1% BSA-PBS with or without 0.1 M monosaccharide solution (D-gal=galactose; D-glu=glucose). Development for the PNA reactivity was with a monoclonal antibody to PNA (153H.1) made in this laboratory and was used at a dilution of 1/300 (ascites) as were the other MAbs used. CH4 is a control IgM MAb of unknown specificity. Epiglycanin was a kind gift of Dr. Carina Henningsson, and HSA was a purified commercial preparation (Sigma). Results are the means and standard errors ( ) of quadruplicates.

Table 18. A Normal AB Serum Contains Naturally Occurring Antibodies to Purified Peak B

	Specific O.D. 405 nm		
	AFP-BP[a] (167H.4 Affinity Chromatography)	AFP-BP[b] (Peak B)	Epiglycanin
AB serum, anti-IgG	.281 (.011)	.977 (.066)	.255 (.030)
PBS, anti-IgG	.107 (.020)	.177 (.011)	.014 (.006)
AB serum, anti-IgM	.197 (.008)	.970 (.098)	.574 (.144)
PBS, anti-IgM	ND	.018 (.005)	.007 (.002)

ELISA results are the means and standard errors ( ) of quadruplicates. Epiglycanin serves as a positive control since it contains TF and Tn antigens.

a) This AFP-BP was isolated from a pool of breast cancer membrane extracted antigens by affinity chromatography on a 167H.4 MAb affinity-sepharose column and was acid eluted.

b) This AFP-BP is purified Peak B isolated by the techniques in Figures 17-18 from solubilized breast cancer membranes.

ND = Not Done

Table 19. The Anti-AFP/AFP-BP Rabbit Serum Does Not Cross-React With Glycophorin or Desialyated Glycophorin

	O.D. 405 nm			
	Glycophorin	N'ase Glycophorin	AFP [c]	Gelatin [d]
NRS	.068 (.010)	.109 (0.28)	.115 (.019)	.057 (.014)
Anti-AFP/AFP-BP [a]	.064 (.009)	.058 (.003)	.336 (.031)	.049 (.009)
Absorbed Serum [b]	.055 (.003)	.059 (.006)	.224 (.034)	.044 (.009)

The ELISA was conducted as in Table 6 except the rabbit antiserum was used at 1/1000. The neuraminidase treatment of glycophorin was performed in the plates as described in the Materials and Methods. AFP is included here as a positive control antigen for the Anti-AFP/AFP-BP serum.

a) The rabbit antiserum made to the crude AFP/AFP-BP described in the Materials and Methods.

b) The E. coli absorbed rabbit antiserum prepared for the cloning procedure.

c) Ultrapure AFP commercially obtained from Dr. Wu.

d) The gelatin used to block the plates (1%-PBS).

Table 20. Purified Human AFP Reacts with Commercial Anti-AFP Serum.

	ELISA O.D. 405 nm	
	Anti-AFP Serum	Normal Rabbit Serum
AFP [a]	2.112 (.032)	.134 (.004)
AFP [b] Exp. #1	2.031 (.060)	.063 (.012)
" Exp. #2	1.548 (.029)	.050 (.005)
" Exp. #3	0.691 (.054)	.016 (.002)

The commercial anti-AFP rabbit antiserum and normal rabbit antiserum were diluted 1/500 for this assay. A goat anti-rabbit IgG (Biorad) was used at 1/1000. Results are the means and standard errors ( ) of triplicates.

a) AFP purified and used in cellular AFP binding inhibition assay.

b) AFP commercially prepared and pure (from Dr. Wu).

Table 21. Pure AFP Bears Cryptic PNA Reactive Sites as Measured by ELISA.

	O.D. 405 nm			
	PNA	Control	PNA Plus Gal	PNA plus GluNAc
ANTIGEN:				
AFP	0.044 (.010)	.046 (.007)	.037 (.008)	0.040 (.003)
Epi	0.673 (.030)	.043 (.009)	.292 (.030)	0.702 (.014)
BSA	0.024 (.003)	.029 (.012)	.027 (.001)	0.024 (.001)
N'ase AFP	0.693 (.035)	.142 (.012)	.150 (.007)	0.372 (.024)
N'ase Epi	1.345 (.010)	.123 (.003)	.795 (.020)	1.328 (.020)
N'ase BSA	0.289 (.018)	.136 (.038)	.186 (.059)	0.159 (.053)

Plates were incubated overnight with 1 ug per well of antigen. Plates were blocked with 1% BSA-PBS. For neuraminidase treatment (N'ase), 0.01 unit/100 ul was added per well and incubated for 3 hours at 37°C in RPMI. After washing five times, the plates were processed as usual for the peroxidase ELISA. PNA (1 mg/ml) was diluted 1/200, the anti-PNA MAb (153H.1) was diluted 1/300 in 1% BSA-PBS. Pure, commercial AFP (Wu, 1984) was tested. Results are expressed as the mean of triplicates and with the standard errors ( ). Gal=2 mg/ml D-galactose; GluNAc=2 mg/ml D-N-acetyl-Glucosamine. The carbohydrate inhibition was done during the incubation with the PNA lectin. Control is the anti-PNA MAb, 153H.1 without prior incubation with PNA.

Table 22. Summary of FACS Analysis on Human PBMC

Experiment #	Cells Tested	Specific % [a] Population Positive for:
1	3 day cultured T cells	98% B_2 microglobulin 0% 167H.1 0% 167H.4 0% 7H.3
	3 day Con A stimulated T cells	98% B_2 microglobulin 0% 167H.1 0% 167H.4 30% 7H.3
2	3 day cultured T cells	96% B_2 microglobulin 0% 167H.1 0% 167H.4 5% 7H.3
3	Freshly isolated PBMC	76% CD3 3% MAC-1 3% BA-1 3% 167H.1
4	T cell line propagated with IL-2 for several months	60% CD4 40% CD8 0% 167H.1
5	3 day, Anti-CD3 activated PBMC	93% CD3 5% BA-1 0% 167H.1
6	2 week old cultured adherent cells	2% 167H.1
7	3 day, PHA activated PBMC	34% 7H.3 18% 50H.19 4% 167H.1
	3 day, cultured PBMC	15% 7H.3 6% 50H.19 0% 167H.1

continued on next page...

Table 22. (continued)

Experiment #	Cells Tested	Specific % [a] Population Positive for:
8	Freshly isolated adherent cells	100% B_2 microglobulin 69% PNA 50% 49H.8 21% 167H.1
	Freshly isolated non-adherent cells	100% B_2 microglobulin 7% PNA 0% 49H.8 0% 167H.1
9	2 day, PHA activated PBMC	53% T4 38% T8 36% 7H.3 15% PNA 0% 167H.1

Experiments were performed on individual blood donors. 7H.3 is anti-Class II MHC MAb. 50H.19 (CD9) is an activation marker of T and B cells. CD3 is a marker for T cells as are T4, and T8. MAC-1 is a monocyte marker, and BA-1 is a B cell marker. 9H.1 is a MAb for B_2 microglobulin.

a) The isotyped matched relevant control MAb has been subtracted.

Table 23. Expression of AFP-BP on PBMC Subpopulations by FACSCAN

Experiment #	Condition		% Positive [a] Lymphocytes [b]	Monocytes [c]
1	Freshly isolated	167H.1	14%	14%
		167H.4	8%	7%
		CD3	55%	10%
2	2 day unactivated	167H.1	1%	7%
		167H.4	1%	4%
		7H.3	21%	70%
		9H.1	98%	100%
	2 day Con A activated (10 ug/ml)	167H.1	1%	0%
		167H.4	1%	0%
		7H.3	35%	46%
		9H.1	83%	96%
	2 day PHA stimulated (1% Gibco)	167H.1	2%	1%
		167H.4	2%	4%
		7H.3	36%	39%
		9H.1	84%	97%
3	3 day unactivated	167H.1	2%	14%
		167H.2	0%	22%
		167H.3	0%	40%
		167H.4	23%	17%
		Anti-AFP	3%	34%
		7H.3	14%	43%
	3 day PHA treated (20 ug/ml)	167H.1	3%	26%
		167H.2	2%	31%
		167H.3	4%	32%
		167H.4	7%	25%
		Anti-AFP	2%	14%
		7H.3	36%	22%

Experiments were performed on individual blood donors.

a) % positive by FACSCAN after subtraction of relevant isotype control MAbs.
167H.3 is an IgM MAb reactive to unknown breast cancer antigens.
7H.3 is a MAb to human Class II MHC non-polymorphic epitopes.
9H.1 is a MAb to human Class I MHC non-polymorphic determinants.
Anti-AFP is a commercially available MAb to human AFP.

b) Gating was on the smaller, less granular homogeneous subpopulation as determined by both forward and side scatter profiles.

c) Gating was on the larger, more granular homogeneous subpopulation as determined by both forward and side scatter profiles.

Table 24. Inhibition of Human MLR ^{3}H-Thymidine Incorporation by AFP or MAbs to AFP-BP

	^{3}H-THYMIDINE INCORPORATION (cpm)	INHIBITION
DONOR B RESPONDING TO DONOR A:		
BαB	2222± 470	-
BαA	21634±1113	-
BαA + 167H.1 (50 ug/ml)	12049± 943	44%*
BαA + 167H.4 (50 ug/ml)	12554±1152	42%**
BαA + Crude AFP (50 ug/ml)	10541±1267	51%***
BαA + IgM Control (50 ug/ml)	19194±1722	-
DONOR A RESPONDING TO DONOR B:		
AαA	2232± 837	-
AαB	23304±2367	-
AαB + 167H.1 (50 ug/ml)	13169±2589	43%***
AαB + 167H.4 (50 ug/ml)	16456±1545	29%*
AαB + Crude AFP (50 ug/ml)	5924± 526	75%*
AαB + IgM Control (50 ug/ml)	23226± 994	-

The results for the one-way Mixed Lymphocyte Reaction (MLR) are shown as the means and standard errors of triplicates. The % inhibition was calculated based on the control values without added proteins. Assay was conducted with 10% (heat inactivated) normal AB serum and the donors were random donors (O blood group) from the Red Cross, Blood Transfusion Service (Edmonton). Crude AFP refers to the pre-albumin peak from HTP columns which contains AFP derived from PE. The control IgM MAb was 167H.3 which reacts with human PBMC (Table 23).

*$p<.005$
**$p<.01$
***$p<.0005$

Table 25. The Anti-AFP-BP MAbs Inhibit the Proliferation of P388-AD2 Cells as Assessed by the MTT Assay.

	SPECIFIC O.D. 570 nm	% INHIBITION	p Values
Control	.821±.083	0%	
167H.1 (50 ug/ml)	.314±.026	62%	p<.0005
167H.1 (25 ug/ml)	.415±.045	49%	p<.005
167H.4 (50 ug/ml)	.395±.039	52%	p<.01
167H.4 (25 ug/ml)	.411±.035	50%	p<.005
49H.8 (50 ug/ml)	.741±.054	10%	NS
49H.8 (25 ug/ml)	.711±.080	13%	NS
CH4 (50 ug/ml)	.824±.060	0%	NS
CH4 (25 ug/ml)	.792±.036	4%	NS

Results are expressed as the mean and standard error of quadruplicates as measured by the MTT assay at an O.D. of 570 nm. This assay was conducted for 48 hours in 1% Ultrasor-RPMI Media. % inhibition is standardized to the control (100%) without any added proteins. CH4 and 49H.8 are control IgM (purified) MAbs. Similar results were obtained by ^{3}H-thymidine incorporation (Dr. Mary Crainie, personal communication).

NS = Not Significant.

Table 26. Effect of AFP and/or Anti-AFP-BP MAbs on the Proliferation of LoVo Cells

	Specific O.D. 595 nm	% Control	P Values
Control	.089±.028	100%	
HSA (50 ug/ml)	.081±.032	91%	
AFP (50 ug/ml)	.145±.024	163%	$p<.005$ [a]
49H.8 (50 ug/ml)	.067±.016	75%	NS
49H.8 (10 ug/ml)	.068±.011	76%	NS
167H.4 (50 ug/ml)	.051±.014	58%	$p<.02$ [a]
167H.4 (10 ug/ml)	.033±.020	37%	
167H.1 (50 ug/ml)	.026±.018	29%	$p<.02$ [a]
167H.1 (10 ug/ml)	.022±.011	25%	
AFP + 49H.8 (50 ug/ml)	.124±.015	139%	NS
AFP + 49H.8 (10 ug/ml)	.154±.016	173%	NS
AFP + 167H.4 (50 ug/ml)	.093±.028	104%	NS
AFP + 167H.4 (10 ug/ml)	.141±.032	158%	NS
AFP + 167H.1 (50 ug/ml)	.083±.012	93%	$p<.01$ [b]
AFP + 167H.1 (10 ug/ml)	.073±.017	82%	$p<.0005$ [b]

Results are the means and standard errors of quadruplicates of MTT reduction in viable cells at 48 hours in IMDM media which contains transferrin, insulin and fatty acids.

a) when compared to control values.

b) when compared to AFP (alone) values.

Table 27. AFP or the 167H.1 MAb Enhances the Growth of HL-60 Cells

	^{3}H-Thymidine Incorporation (cpm)		
	48 hours	72 hours	96 hours
Control	1930 (628)	306 (55)	140 (14)
AFP	9716 (715)	10243 (217)	12214 (62)
167H.1 MAb	7590 (172)	7559 (37)	9581 (103)
AFP + 167H.1 MAb	13670 (1114)	19348 (344)	13153 (809)

Results are the means and standard errors ( ) of triplicates, and were conducted in serum-free IMDM media containing transferrin, insulin and fatty acids. Final concentration of the purified proteins were: AFP - 10 ug/ml, 167H.1 - 10 ug/ml. $2x10^4$ cells per well in 96 well Linbro plates were used. AFP was the one used for the cellular binding inhibition experiments as shown in Tables 4 and 5.

Results supplied by Dr. Simeon Vassiliadis.

Table 28. Comparisons of Dyes for Dye Exclusion Viability Staining for Apoptosis Induction.

	PREFERENTIAL UPTAKE OF DYES [a]	
	FITC [b]	Propidium Iodide or Trypan Blue
Viable Cells	-	-
Blebbing Cells	+	-
Viable Apoptotic Cells	++	-
Non-Viable Apoptotic Cells	+/-	++
Apoptotic Bodies [c]	-	-

a) A compiled composite summary of staining patterns of consecutive stages of apoptosis based on the work of Sasaki DT et al, 1987, Martin et al, 1990b, Shi et al, 1989 and thesis investigation (see later).

Note that depending on the phase of death induction analysed (i.e. timing), FITC staining may or may not correspond to propidium iodide (PI) staining. It has been estimated that the time required for viable apoptotic cells to convert to non-viable apoptotic cells such as for the HL-60 cells is 4 hours at 37°C (Martin et al, 1990b). Viable apoptotic cells have condensed chromatin and fragmented DNA (Martin et al, 1990b).

b) By size and FITC uptake as revealed by flow cytometric analysis, blebbing cells can be easily distinguished from apoptotic body formation (see later).

c) Apoptotic bodies (cellular debris) are easily visualized based on small size by FACSCAN morphological parameters but is not included in the interpretation of % viable or % non-viable cells in this thesis.

Table 29. Propidium Iodide Staining is Analogous to Trypan Blue Viability Staining of HL-60 Cells.

	% TOTAL POPULATION [a]				% POSITIVE FOR TRYPAN BLUE
	PI Single Positive	PI/FITC Double Positive	FITC Single Positive	PI/FITC Double Negative	Positive
Passage 10	0.98%	0.20%	8.28%	90.54%	0%
Passage 10 (overgrown)	7.00%	6.08%	28.90%	58.02%	NT
Passage 49	1.94%	0.92%	17.96%	79.18%	3%
Passage 49 (overgrown)	1.36%	3.48%	51.18%	43.98%	NT

HL-60 cells at the various passage numbers at $1x10^6$/ml had been cultured in flasks in 10% FCS-RPMI (for 24 hours) or allowed to overgrow for an additional three days wherein the culture media turned acidic (overgrown), then stained with PI (propidium iodide), and FITC and/or trypan blue to determine viability. As expected, viability (PI/FITC double negative) was highest for the lower passage number which was not overgrown and lowest for late passage HL-60 cells which had been overgrown. FITC staining appears to stain dying cells but which may or may not yet be dead as judged by trypan blue. PI staining, in analogy to trypan blue, appears to stain non-viable cells (see Table 28).

a) % of total population as gated by FACSCAN analysis.

Table 30. Late Passage HL-60 Cells are More Sensitive to Programmed Cell Death by Subculture in 96 Well Microtitre Plates Than Early Passage HL-60 Cells.

Passage Number	Culture Period	% VIABLE [a] 10% FCS-RPMI	2% FCS-RPMI
P.10	24 hours	86.8%	29.4%
"	48 hours	94.0%	56.0%
P.49	24 hours	28.7%	4.3%
"	48 hours	7.9%	4.9%

Early passage (P.10) and late passage (P.49), HL-60 cells were cultured at $1x10^5$/well in 96 well microtitre well plates for either 24 or 48 hours, in 10% FCS or 2% FCS containing RPMI media, then double stained for PI and FITC. The results indicate that the late passage HL-60 cells are more susceptible to programmed cell death than early passage numbers as a higher loss of viability occurred at an earlier time and in more enriched media. Results are representative of a single experiment but the finding that early passage HL-60 do not exhibit significant cell death by 48 hours in 5% FCS-RPMI but that late passage numbers do, was found in several experiments.

A comparison of the results obtained here for Passage 49 in 96 well plates wherein HL-60 cells are adherent, to the results in Table 29 wherein HL-60 cells grow non-adherently indicate that significant cell death (28.7% viable) occurs in the 96 well plates compared to flask grown (79.18% viable, Table 29). Since the number of cells per ml and the media are identical, as in the passage number, these results strongly imply that late passage HL-60 cells are sensitive to death induction associated with adherence in the 96 well plates, not found for early passage cells but adherent HL-60 cells.

a) % viable refers to percentage of cells double negative for PI and FITC enhanced uptake.

Table 31. Cell Surface Expression of AFP-Binding Protein Isoforms on HL-60 is Down-Regulated with Passage Number and/or with the Induction of Programmed Cell Death.

Passage #	Condition [b]	% Cells Positive by FACS Analysis [a] 167H.1	167H.4
8	A	79%	66%
8	B at 48 hours with 1.2% DMSO	61%	35%
8	B at 96 hours with 1.2% DMSO	57%	52%
32	A	23%	34%
32	B at 48 hours	4%	0%
32	B at 96 hours	20%	33%
44	A	ND	0%
54	A	0%	0%

a) Backgrounds (IgM control MAb, NSC C1 20) were subtracted.

ND = Not done

b) Condition A = Flask grown in 10% FCS-RPMI (with phenol red); $1x10^6$ cells/ml.

Condition B = Cultured in 96 well Linbro plates in 100 ul of 5% FCS-RPMI (no phenol red); $1x10^6$ cells/ml.

The results show that the down-regulation of the 167H.4 reactive isoform is associated with late passage number versus early (Experiment 1), and with culturing for 48 hours in 96 well plates in 5% FCS-RPMI (Condition B, Experiment 2) in the presence of 1.5% DMSO. However, by 96 hours the levels of expression of the 167H.4 reactive isoform is restored (Experiments 2 and 3). This is consistent with the notion that the death associated cellular induction signals (see Figure 28) induced upon culturing in the 96 well plates (Condition B), are associated with the temporal down-regulation of the 167H.4 isoform.

Table 32. The 167H.1 But Not the 167H.4 MAb Promotes Cell Viability of HL-60 Cells as Determined by FACSCAN Analysis.

	% Viable Cells [a]
IgM control (10 ug/ml)	57.0%
167H.4 (10 ug/ml)	60.0%
167H.1 (10 ug/ml)	98.0%

Late passage HL-60 cells (passage #49) were cultured at $1x10^5$ cells/well in Linbro plates in 5% FCS-RPMI (no phenol red) for 96 hours, then stained with PI and FITC. 98.02% of the cells were viable after 96 hours when cultured with the 167H.1 MAb whereas 60.04% and 57.08% were viable with the 167H.4 MAb or control IgM MAb (NSC C1 20), respectively.

a) % viable refers to cells which are double negative for PI/FITC uptake by FACSCAN analysis.

Table 33. The Growth Enhancement by the 167H.1 MAb of HL-60 Cells in Suboptimal Media Does Not Exceed Control Levels in Optimum Media.

		^{3}H-Thymidine Incorporation ($\times 10^{-3}$)	
		10% FCS-RPMI	1% Ultrasor-RPMI
Passage 10	Media Control	588	462
	167H.1 MAb	506	541
	167H.4 MAb	576	446
	IgM Control MAb	622	489
Passage 49	Media Control	363	288
	167H.1 MAb	429	363
	167H.4 MAb	406	279
	IgM Control MAb	488	289

Results expressed as the means of triplicates where variance was less than 10% for $1x10^{5}$ cells per well where young (Passage 10) HL-60 cells were compared to more senescent (Passage 49) HL-60 cells in 96 well plates at 48 hours. Purified MAbs were used at 5 ug/ml. The control IgM MAb was NSC Cl 20. The results show that on a per cell basis, senescent HL-60 cells incorporate less thymidine than younger HL-60 cells and that the enhancement of thymidine incorporation by the 167H.1 MAb in suboptimal culture conditions (1% Ultrasor) does not exceed control levels in optimal culture conditions (10% FCS-RPMI). This may be consistent with the notion that the 167H.1 MAb may not induce a growth signal per se to HL-60 cells, but restores viability (or abrogates programmed cell death). It is also consistent with the notion that as HL-60 cells age in culture there is a loss of proliferative capacity.

Table 34. AFP Blocks Death Induction in Late Passage HL-60 Cells.

	% Non-Viable Cells [a]		
Medium	FITC Uptake	PI Uptake	48 Hour ^{3}-H-Thymidine [b] (cpm)
5% FCS-RPMI	78%	88%	76,035
" + AFP (60 ug/ml)	31%	33%	95,841

HL-60 cells (passage 50) were subcultured in 5% FCS-RPMI (no phenol red) at $1x10^5$/well in 96 well Linbro plates. The AFP used was the crude HTP isolate also employed in Table 24.

a) % relative FITC or PI uptake was measured at 24 hours as an indication of level of death induction and was analysed by FACSCAN analysis.

b) Results are the means of triplicates wherein the variance was less than 10%.

Table 35. The 167H.1 But Not 167H.4 MAb Blocks Death Induction in Cultured Multinegative Human Thymocytes.

	% Non-Viable Cells Cells Cultured With:			
	Media	167H.1 MAb	167H.4 MAb	IgM Control MAb
Experiment I: [a]	56%	12%	56%	53%
Experiment II: [b]	46%	2%	36%	31%

Human thymocytes were depleted of CD3, CD4 and CD8 antigen expressing cells (prepared by Eva Pruski) and cultured at $1x10^5$ cells per well in 5% FCS-RPMI (without phenol red) for 7 days in 96 well plates with or without 100 ug/ml of purified IgM MAbs. The cells were stained with both propidium iodide and FITC as outlined in Materials and Methods. Cells were gated on the number of double positive brightly staining cells by FACSCAN analysis, as a double indication of non-viable cells. Results are representative of 4 experiments, and suggest that the 167H.1 MAb but not the 167H.4 and control IgM (NSC C1 20) MAbs inhibits death induction (or promotes viability) in developing human thymocytes.

a) Experiment I was conducted on anti-CD3 coated (1 ug/well) 96 well plates.

b) Experiment II was conducted with uncoated wells in the 96 well plates.

Table 36. DMSO Induces a Loss of HL-60 Viability Reversed by the 167H.1 MAb.

	% NON-VIABLE CELLS [a]		
	24 Hours	48 Hours	72 Hours
1.2% DMSO	20.9%	31.3%	47.3%
1.2% DMSO plus 167H.1 MAb (10 ug/ml)	15.5%	10.1%	7.5%

HL-60 cells (Passage number 32) were cultured in 5% FCS-RPMI at $1x10^5$ cells/well in 96 well Linbro plates then stained with FITC and analysed by FACSCAN. DMSO treatment was found to morphologically change the appearance of HL-60 cells from round to a fibroblast-like morphology not reversed by the 167H.1 MAb.

a) % non-viable refers to the percentage of cells displaying an increase in FITC uptake as analysed by FACSCAN analysis.

Table 37. The UCHL-1 MAb but not Other MAbs to the CD45 Antigen Activate Death Induction in HL-60 Cells: Rescue is Promoted by the 167H.1 but not the 167H.4 MAb.

	% VIABLE CELLS [a]
Media Control	26%
Anti-CD45RO	6%
Anti-CD45RA	33%
Anti-CD45	47%
Anti-CD45RO plus 1 ug/ml 167H.1	7%
Anti-CD45RO plus 10 ug/ml 167H.1	29%
Anti-CD45RO plus 1 ug/ml 167H.4	1%
Anti-CD45RO plus 10 ug/ml 167H.4	9%
Anti-CD45RO plus 1 ug/ml IgM Control	8%
Anti-CD45RO plus 10 ug/ml IgM Control	5%

Medium passage HL-60 cells (Passage 32) were incubated for 96 hours in 96 well plates in 5% FCS-RPMI, then stained with FITC. Control IgM MAb was NSC Cl 20. For anti-CD45RO treatment 30 ug/ml of the UCHL-1 MAb (IgG2a) was used. For anti-CD45RA treatment 10 ul of FMC-71 Mab (IgG_1) supernatant was added to wells (100 ul). For anti-CD45 treatment, 30 ug/ml of the ASH1621 MAb (IgG2a) was used.

a) % viable cells refers to % of cells which do not exhibit enhanced FITC uptake by FACSCAN analysis.

Table 38. Ratio of Stimulation of Phosphorylation of the Rabbit Anti-AFP/AFP-BP Immunoprecipitated Proteins.

	cpm P^{32}	Ratio of Stimulation
Triggered with:		
167H.1 MAb (200 ug)	32,966	14:1
CH.4 Control (200 ug)	2,271	

The "in vivo" kinase experiment (see Materials and Methods) was conducted on 1.25×10^7 cells which were pre-labeled with 0.5 mCi of P^{32} for 1.5 hours. The cells were then triggered with either 200 ug of 167H.1 MAb or the control CH.4 MAb and incubated for 20 minutes at 37°C. After stopping the reaction and washing the cells (see Materials and Methods), the immunoprecipitates, conducted with the rabbit anti-serum to the crude AFP/AFP-BP were performed on pre-cleared cell lysates, and a sepharose conjugated goat anti-rabbit IgG was used for the immunoprecipitation. After five washes, the radioactivity remaining in the tube was counted.

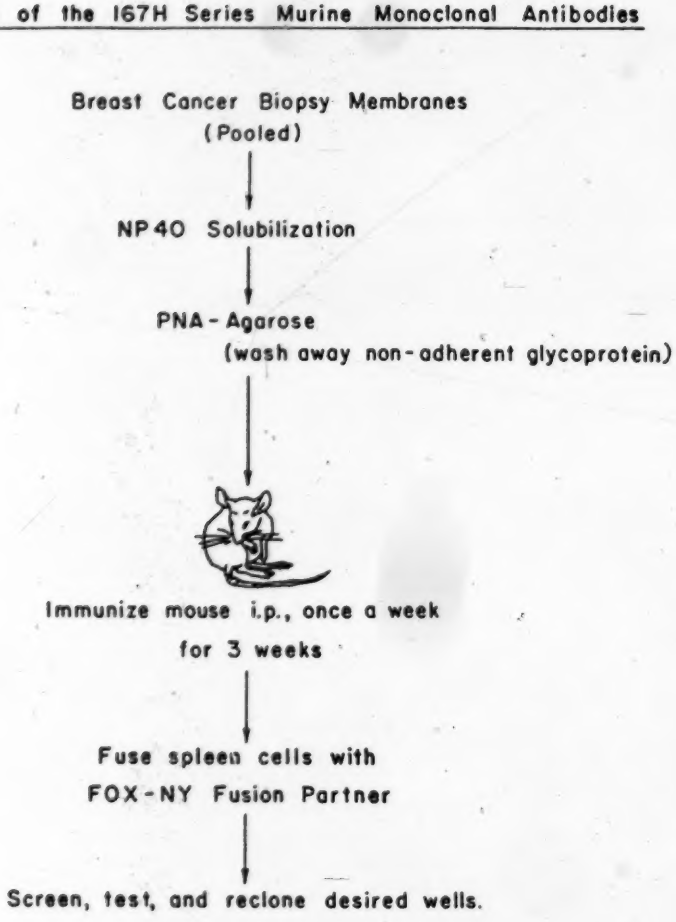

Generation of the 167H Series Murine Monoclonal Antibodies
Breast Cancer Biopsy Membranes
(Pooled)
NP40 Solubilization
PNA - Agarose
(wash away non-adherent glycoprotein)
Immunize mouse i.p., once a week
for 3 weeks
Fuse spleen cells with
FOX-NY Fusion Partner
Screen, test, and reclone desired wells.

Figure 1. Schematic Illustration of Fusion Protocol.

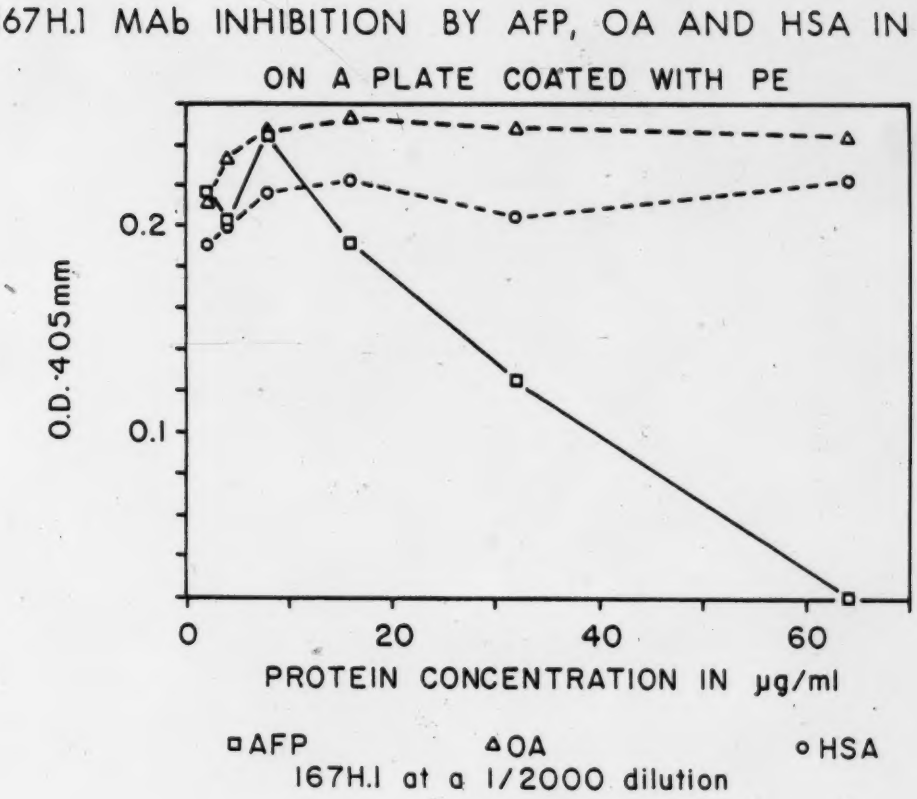


Figure 2. AFP Specifically Inhibits the Binding of 167H.1 to a Soluble Source of the AFP-BP.

PE (a pleural effusion of a lung metastatic breast carcinoma) was diluted 1/30 with PBS and 50 ul was added to plates and incubated overnight. Blocking was with 1%-gel-PBS for 30 minutes. AFP (squares), OA (triangles) or HSA (circles) was tested for their ability to inhibit the binding of the MAb in a one hour assay, and then processed as per the usual ELISA. Results are the means of duplicates and were supplied by Dr. Ricardo Moro.

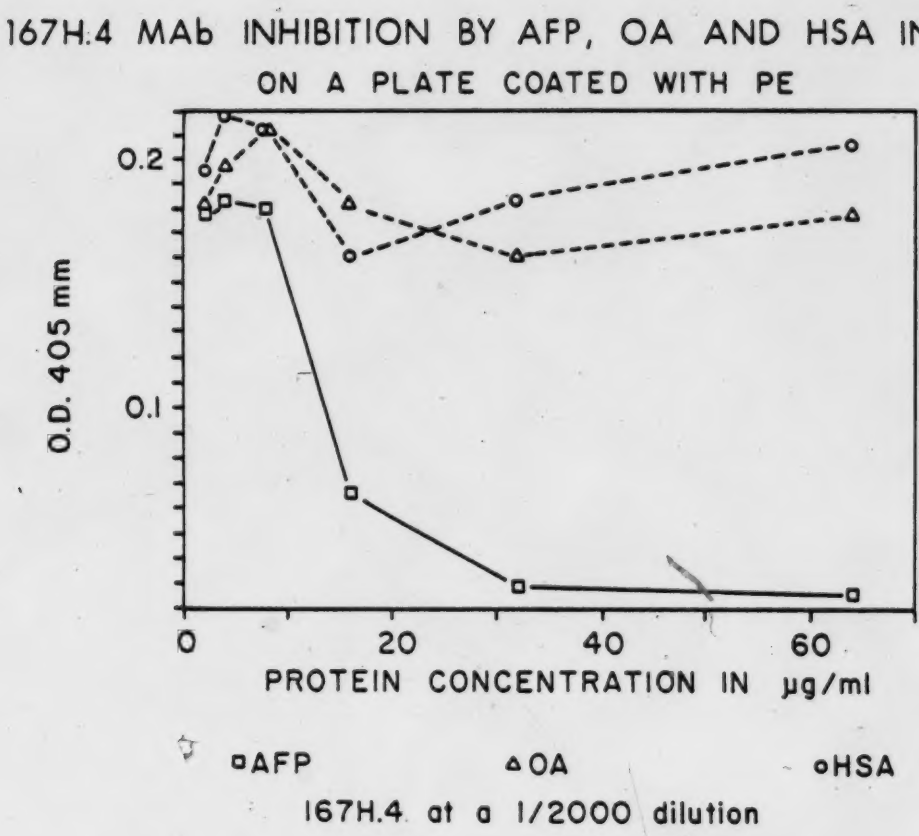


Figure 3. AFP Specifically Inhibits the Binding of the 167H.4 MAb to a Soluble Source of the AFP-BP.

Legend is the same as for Figure 2.

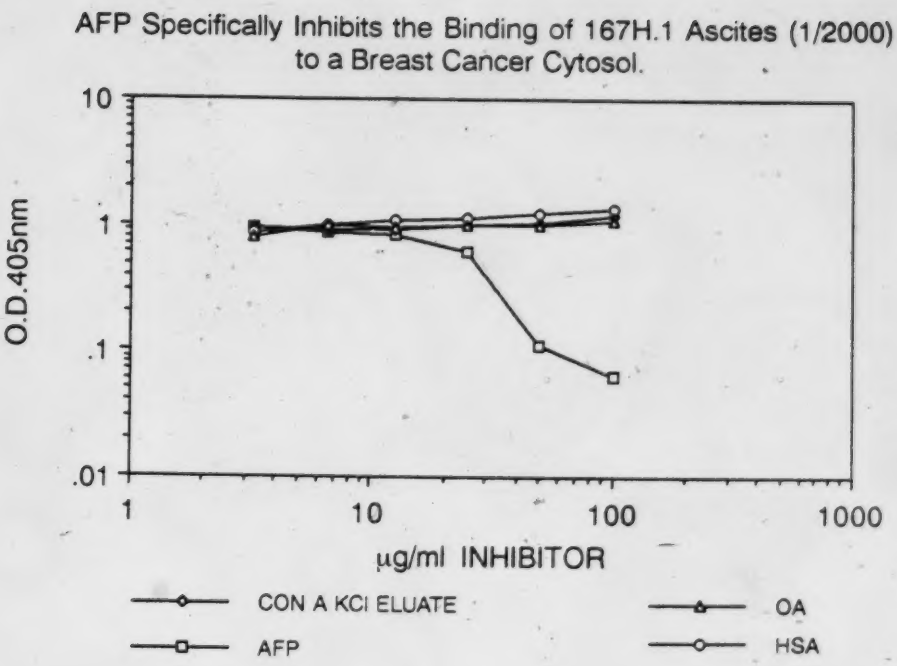


Figure 4. AFP Specifically Inhibits the Binding of the 167H.1 MAb to a Breast Cancer Cytosol, 9250.

Legend is the same as for Figures 2 and 3 except that the cytosol was added at 1 ug/well. Results are the means of duplicates. Variance was less than 10%. ConA KCl eluate refers to a crude preparation of non-AFP containing proteins which was eluted from a Con A affinity column before the specific elution of AFP used in these experiments. See Materials and Methods for further details of ConA affinity purification of HTP derived crude AFP/AFP-BP from PE.

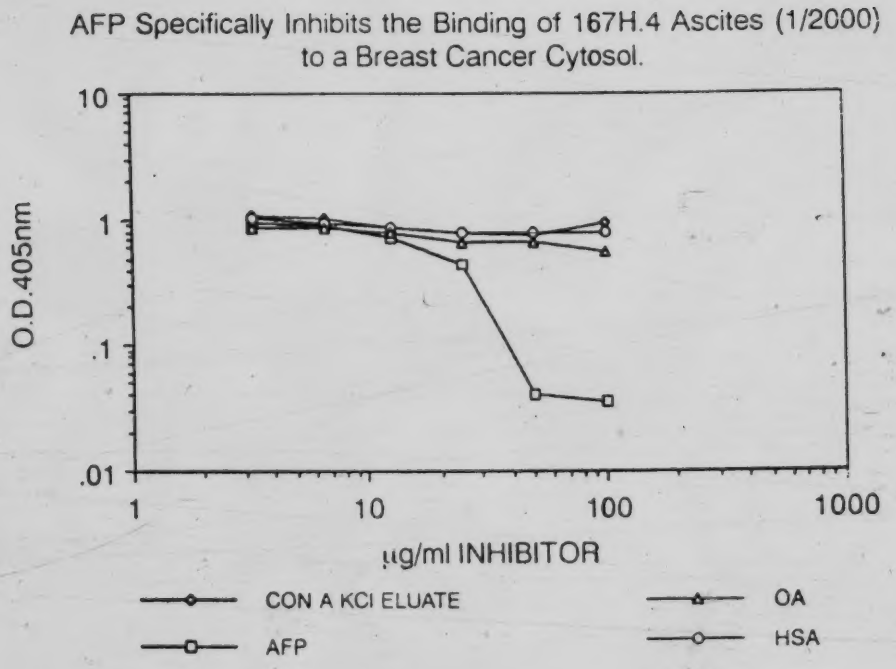


Figure 5. AFP Specifically Inhibits the Binding of the 167H.4 MAb to a Breast Cancer Cytosol, 9250.

Legend is identical to Figure 4.

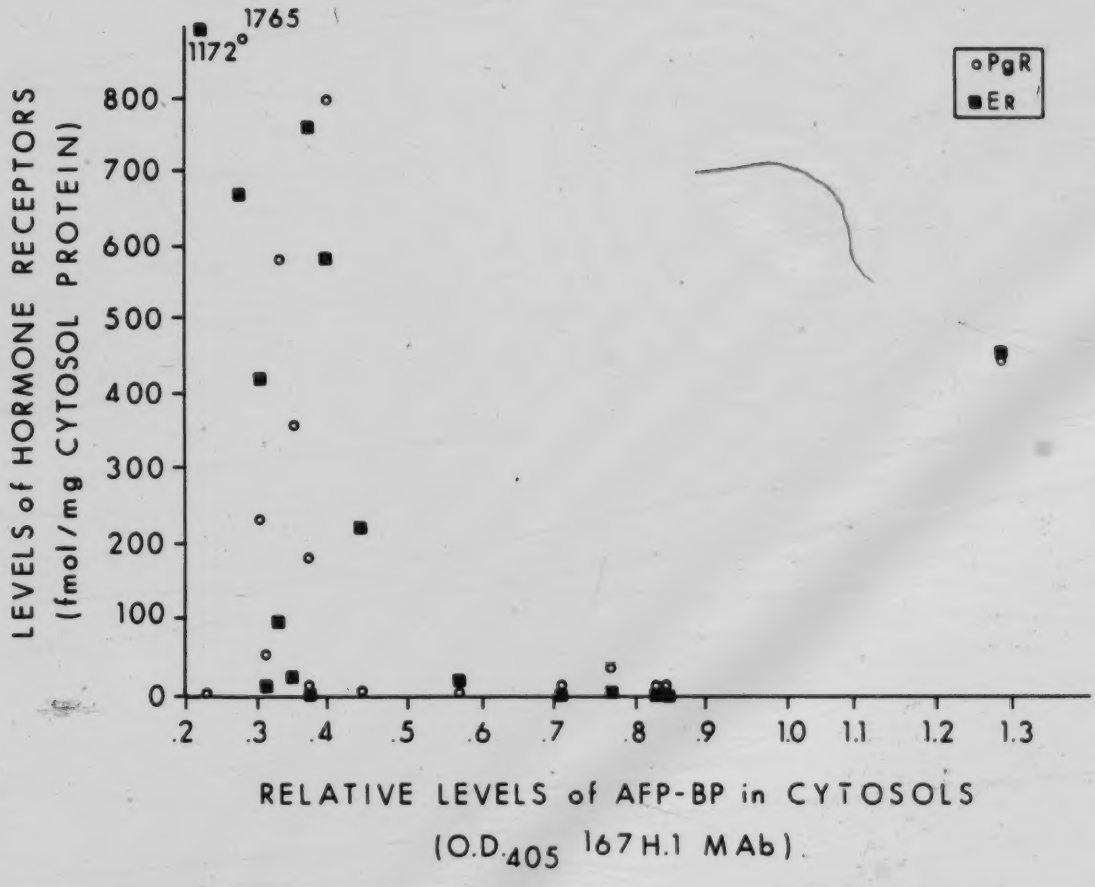


Figure 6. The AFP-BP is Demonstrable in Breast Cancer Cytosols and Shows a Non-linear Inverse Correlation to ER/PgR Status.

Each cytosol was first titrated in a preliminary study to establish maximal 167H.1 binding. Since the cytosols have all been adjusted to 1 mg/ml by the Hormone Receptor Laboratory, it was found that all cytosols showed a maximal reactivity when diluted 1/160 with PBS (.75 ug/well). The 167H.1 was employed in the usual ELISA, and the ER (estrogen receptor) and PgR (Progesterone Receptor) levels were later revealed by Dr. William McBlain (in a blinded fashion). A non-linear inverse correlation of the relative levels of AFP-BP to ER levels (P less than 0.001) and to PgR levels (P less than 0.01) was established by the Mann-Whitney U test, whereas there was no significant correlation of ER to PgR levels established.

Legend for Figure 7.

Blots were performed as outlined in the Materials and Methods and were performed on PE. Gels used were 5% PAGE (native gels). Molecular weight markers for native PAGE gels were obtained from Biorad.

A) Lane 1, 167H.4; Lane 2, 167H.1; Lane 3, 167H.4 on neuraminidase treated nitrocellulose sheets (after blocking); Lane 4, as per lane 3 but with the 167H.1 MAb. Developed by the indirect HRP method (Biorad). The negative control IgM MAb (Y5781.4) did not label any band (data not shown).

B) Autoradiography of binding of ^{125}I-AFP to PE after protein transblotting. The specific activity of the labelled AFP was 12.5 x 10^6 cpm/ug AFP. 50 ug were used to develop the blot.

Lane 1: Neuraminidase treated nitrocellulose (10 units/100 mls of PBS, at 37°C for 2 hours).

Lane 2: Same treatment but lacking neuraminidase.

The blots were washed 7 times (over 4 hours) on a rotating tray before exposing for 1 week at -70°C.

Figure 7. Both ^{125}I-AFP and the Anti-AFP-BP MAbs Recognize a Similar Band by Native PAGE Analysis.

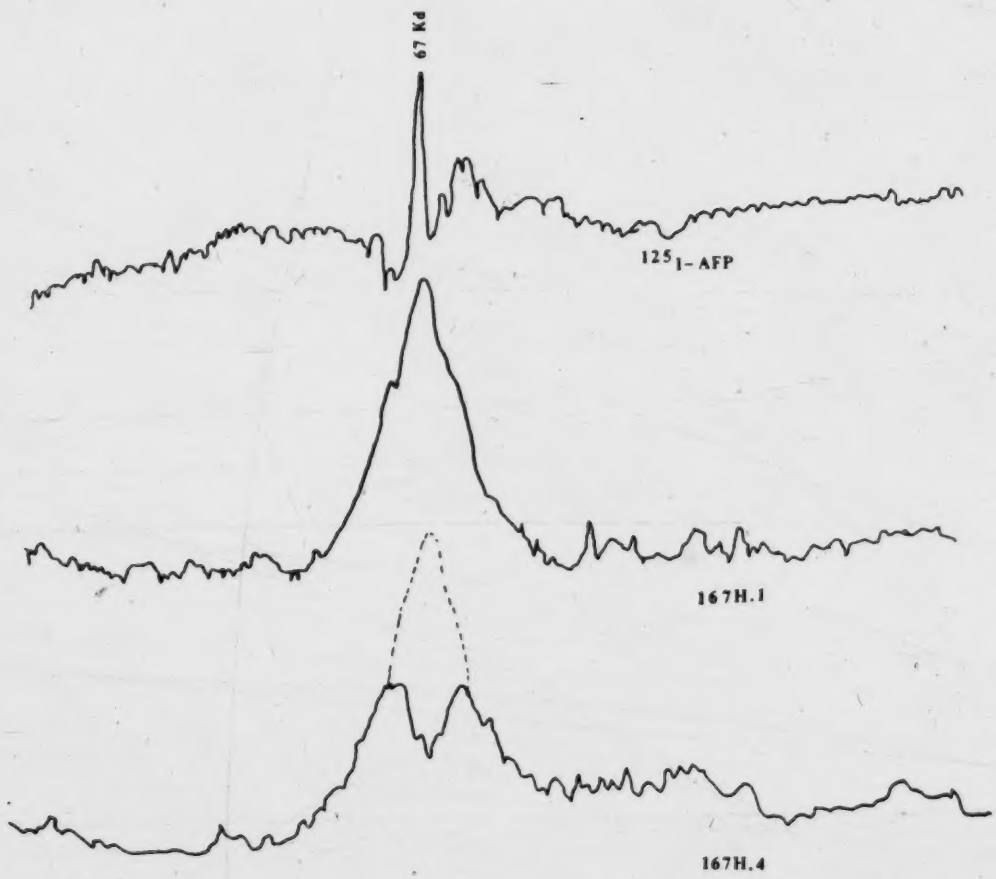


Figure 8. Densitometer Readings of Protein Transblots from 5% PAGE Gels.

The transblots of Figure 7 were scanned by densitometer. Dotted lines for the 167H.4 MAb give an estimate of the DAB which had fallen off by the time the blots were scanned. Molecular weight was estimated by comparison to native PAGE gel molecular weight markers as per Figure 7.

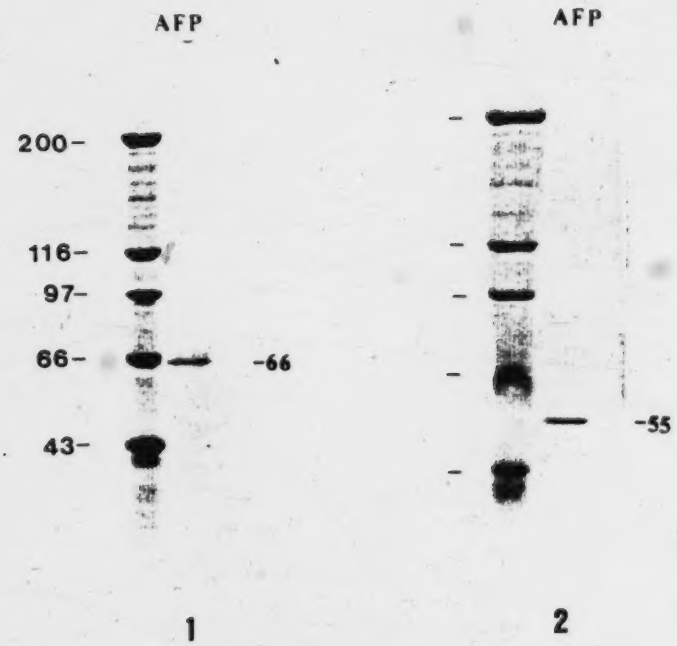


Figure 9. Migration of Purified AFP by 3-15% SDS-PAGE.

Coomassie Blue stained purified AFP (used in the binding inhibition assays).

Gel 1: REDUCING CONDITIONS
Gel 2: NON-REDUCING CONDITIONS

Note that this aberrant behaviour is typical of AFP and the related molecule HSA (i.e. it appears to run at a higher molecular weight of 66 kd under reducing conditions whereas it runs at about 55 kd under non-reducing conditions), and is thought to reflect the high number of disulphide bonds.

Figure 10. 167H.4 Does not React With AFP But Detects a 62/67 Kd Doublet From Breast Cancer Membranes.

Western blot analysis of 167H.4 compared to control IgM (Y5781.4) from 3-15% SDS-PAGE, under non-reducing conditions. T=pooled breast cancer solubilized membranes. 62 = 62 kd 67 = 67 kd. The 167H.4 MAb does not react with purified AFP (lane marked AFP) but identifies a 62/67 kd doublet in the extract of breast cancer tumor membranes (lane marked T).

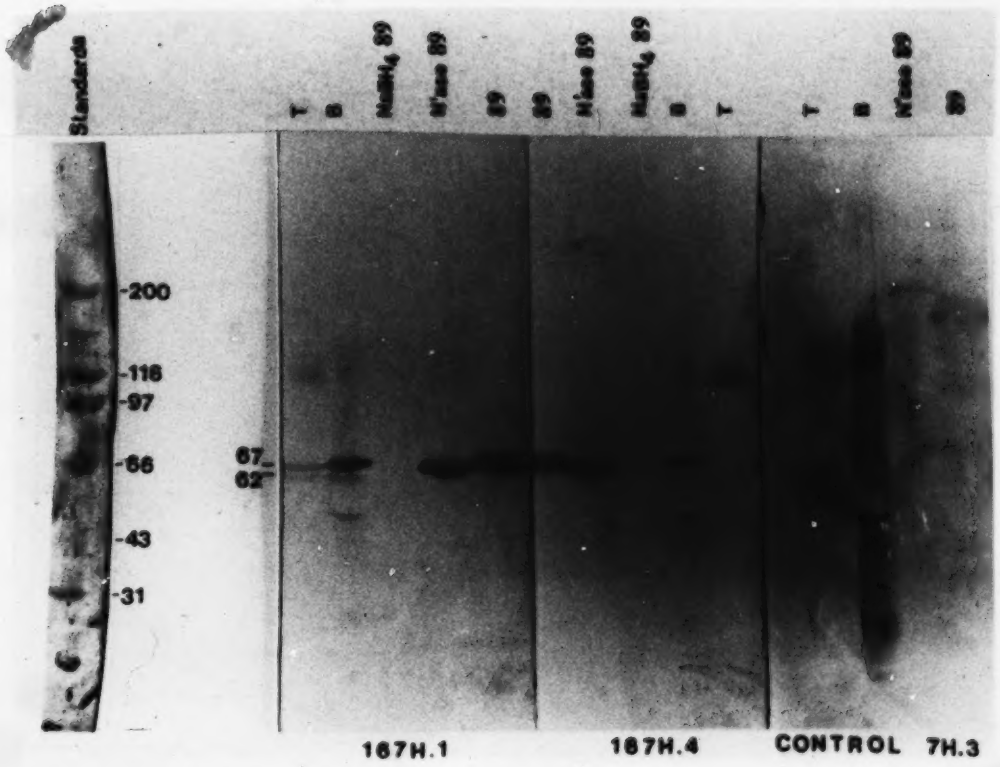


Figure 11. Western Blotting with the Putative Anti-AFP-BP MAbs, 167H.1 and 167H.4. on Soluble, Cellular and Membrane Sources of the AFP-BP.

Western blotting was performed according to Materials and Methods from 3-15% SDS-PAGE gels. Molecular weight standards (Standards) were visualized by Amido Black staining. T = Tumor extract (pooled breast biopsy membranes), B = a single benign breast sample, $NaBH_4$ 89 = Ptnt 89 pretreated with sodium borohydride, N'ase 89 = neuraminidase pretreated Ptnt 89, 89 = Ptnt 89 (Ptnt 89 is a pleural effusion of a lung metastatic breast carcinoma). Numbers refer to molecular weights in kilodaltons. 7H.3 is a MAb which reacts with Class II MHC antigens. The primary antibodies were diluted 1/300 and after washing, the secondary antibody (goat-anti-mouse IgG-alkaline phosphatase, BioRad) was diluted 1/300. Development was with the NBT:BCIP reagent of BioRad.

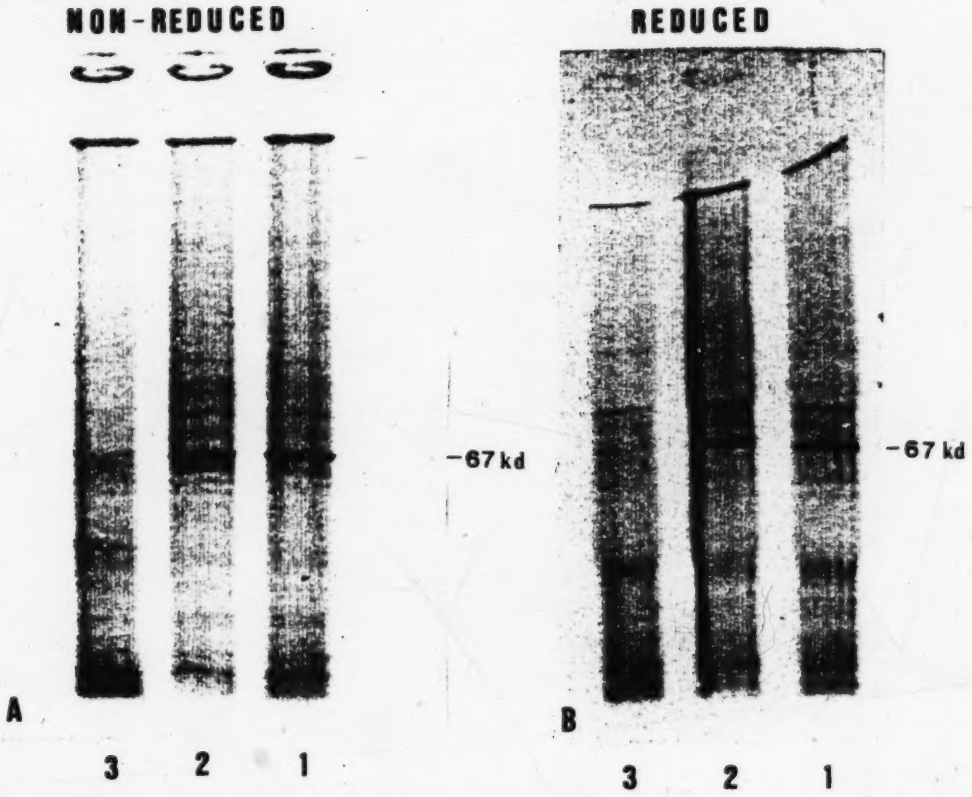


Figure 12. Autoradiography of ^{35}S-Methionine Labeled HL-60 Cells Immunoprecipitated with MAbs to the AFP-BP.

HL-60 cells (human pro-myelo-monocytic) immunoprecipitated with the anti-AFP-BP MAbs were intrinsically labeled according to the Materials and Methods, and revealed by autoradiography. From 10-15% SDS-PAGE Phast-gels (Pharmacia).

A) NON-REDUCING CONDITIONS, B) REDUCING CONDITIONS

Lane 1: 167H.1 MAb, Anti-AFP-BP
Lane 2: 167H.4 MAb, Anti-AFP-BP
Lane 3: CH4 IgM Control MAb

Autoradiographs were exposed for 5 days at -70°C.

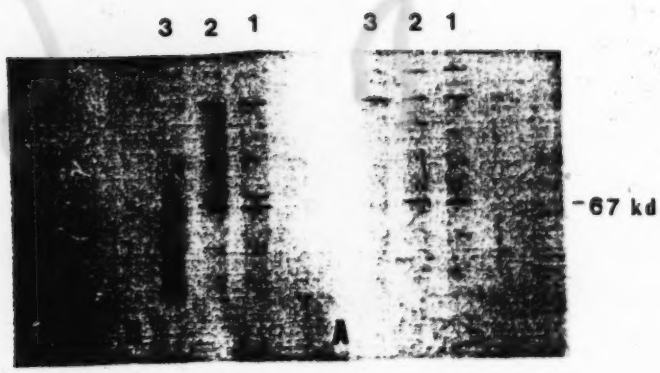


Figure 13. Autoradiography of ^{35}S-Methionine Labeled P388-AD2 Cells Immunoprecipitated with MAbs to the AFP-BP.

Legend is the same as for Figure 12 except the cells used are P388-AD2, a murine dendritic-like cell line.

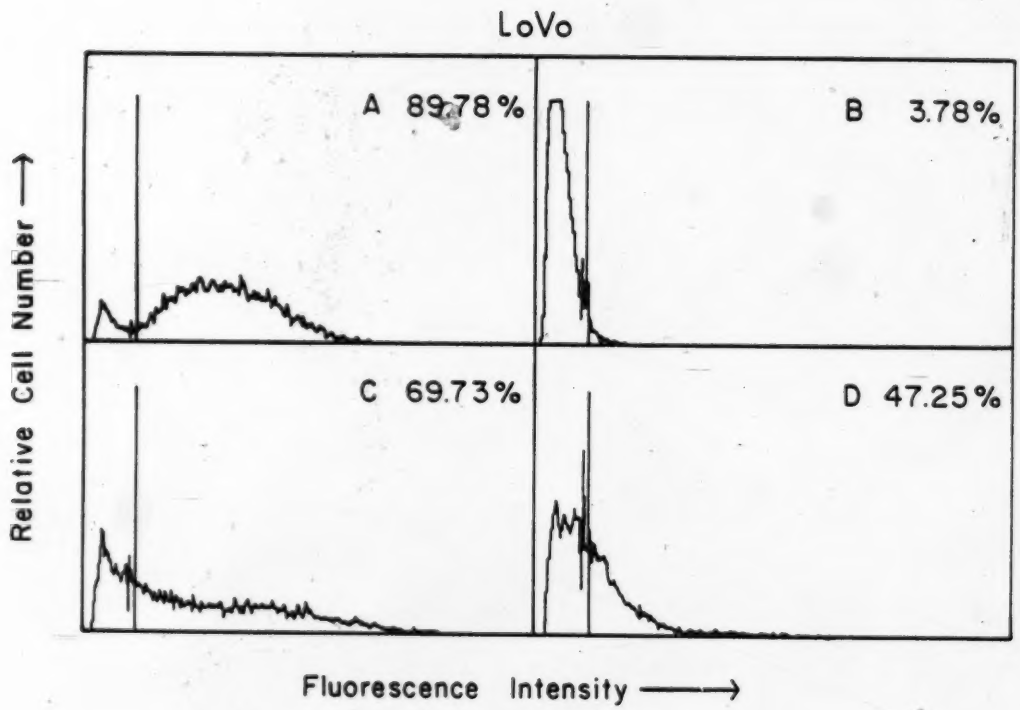


Figure 14. FACS Analysis on LoVo Cell Line.

As outlined in the Materials and Methods the LoVo cell line (a human colorectal carcinoma) was stained with the PNA lectin (A), control MAb, 153H.1, anti-PNA (B), the 167H.1 MAb, (C) and the 167H.4 MAb (D).

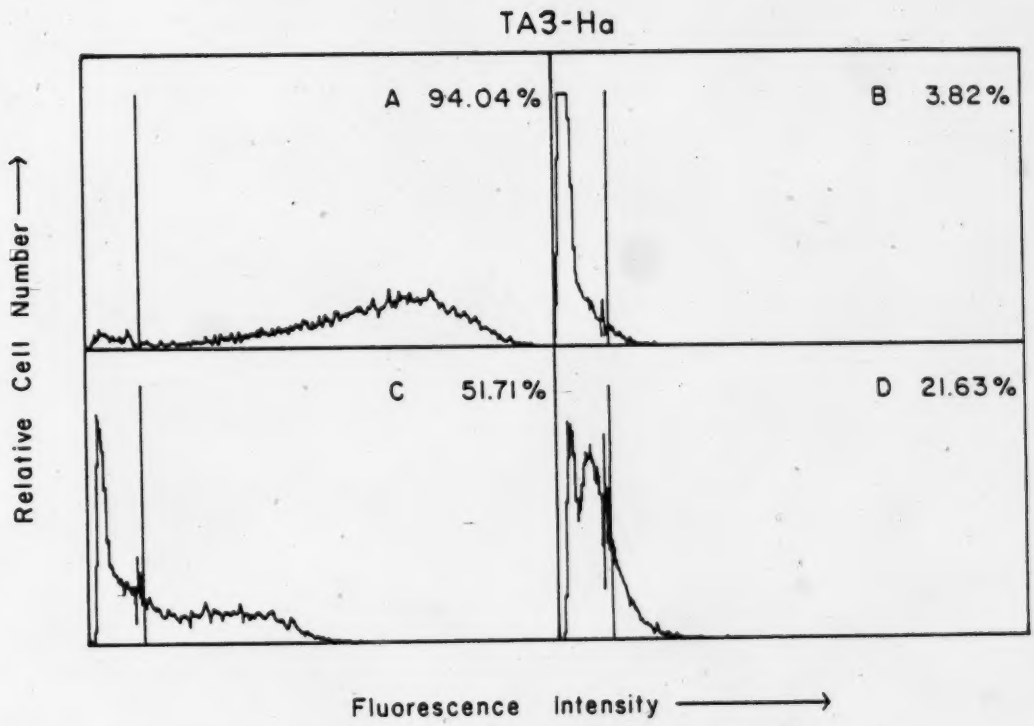


Figure 15. FACS Analysis on TA3-Ha Cell Line.

Legend is the same as Figure 14, but the cells used were the TA3-Ha line, a murine mammary adenocarcinoma.

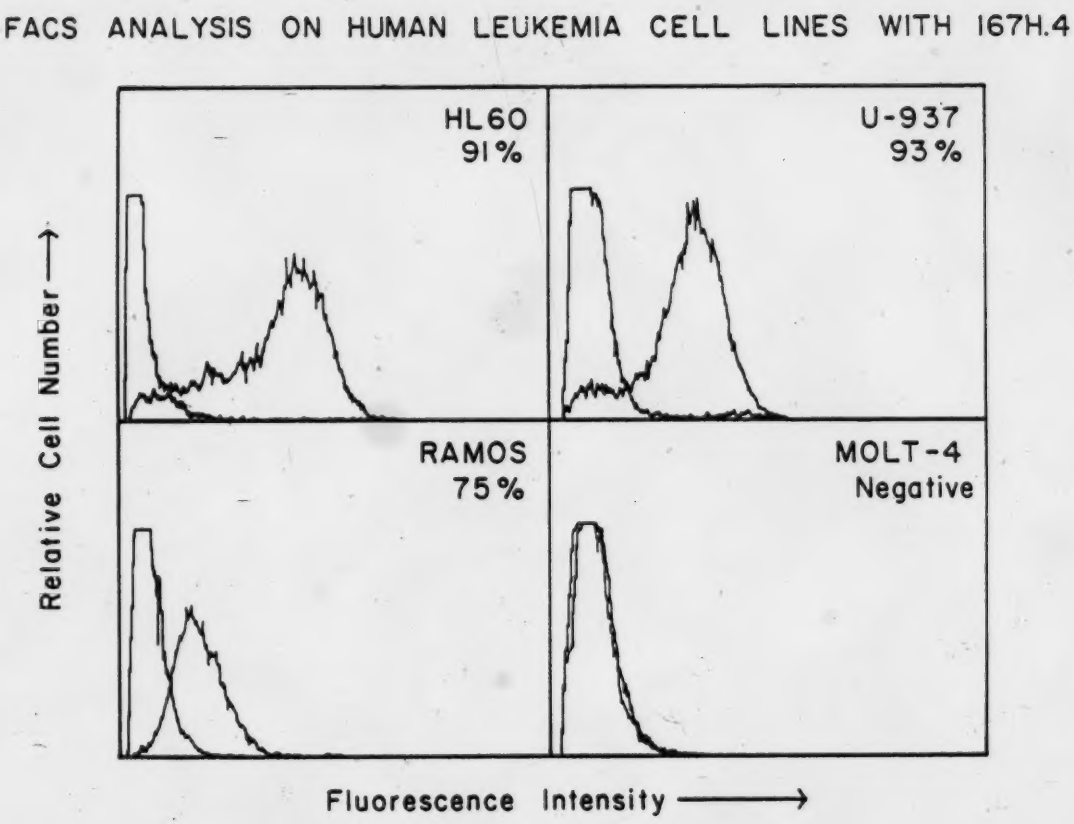


Figure 16. FACS Analysis on Human Leukemic Cell Lines with the 167H.4 MAb.

Performed as outlined in the Materials and Methods, the various human leukemic cell lines were stained with the 167H.4 MAb, and the background control representative of various MAbs is overlayed. Note that Molt-4, is not positive.

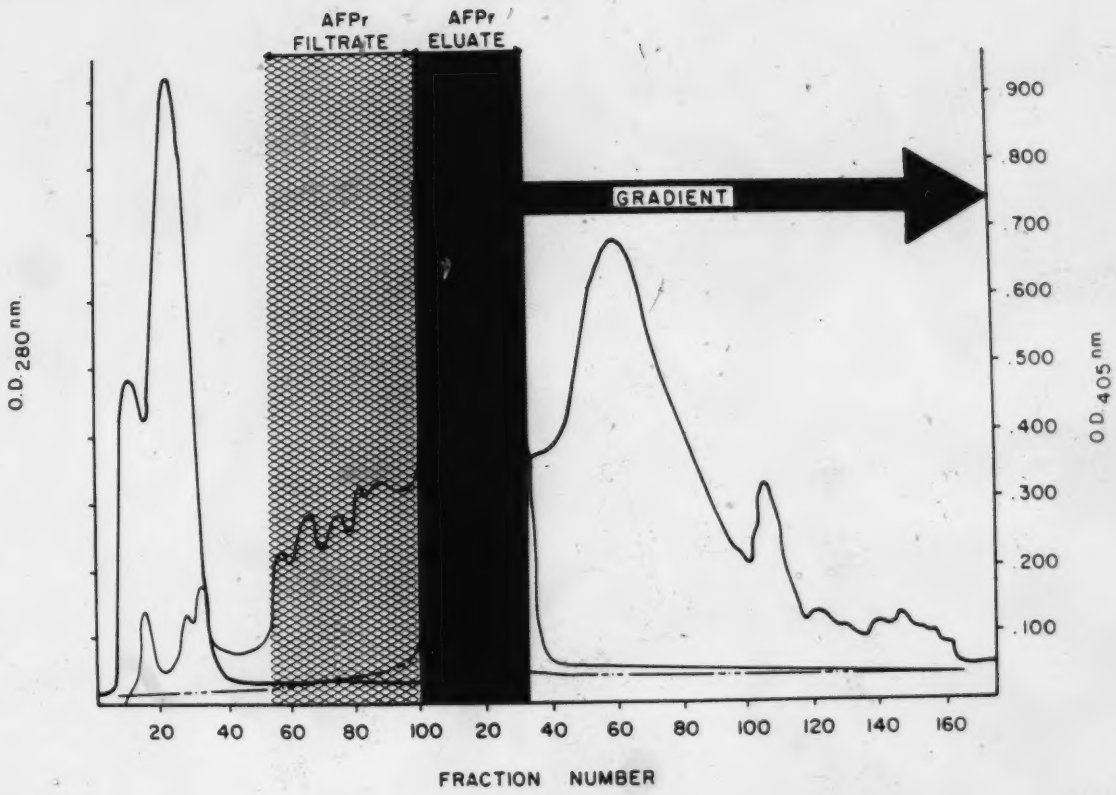


Figure 17. Hydroxylapatite Chromatography of PE and Elution of the AFP-BP.

Fifteen mls of the pleural effusion was diluted 1:1 with a 0.01 M NaH_2PO_4/Na_2HPO_4 (Solution A) and loaded onto the column. 200 mls of Solution A in 2 mls fractions were collected, and then a gradient with a 0.30 M solution of the above (Solution B) was run through the column and again, 2 mls fractions were collected. The fractions were tested by ELISA as described in Materials and Methods. Thick line = A_{280}. Thin line = 167H.4 ELISA reactivity as measured at O.D. 405 nm. Broken line = CH.4 (Control IgM) ELISA reactivity. Those fractions showing the presence of the AFP-BP as measured by ELISA were pooled, concentrated, and dialysed as either an "AFPr Filtrate" or "AFPr Eluate" and further resolved by FPLC-Mono-Q as outlined in Figure 18.

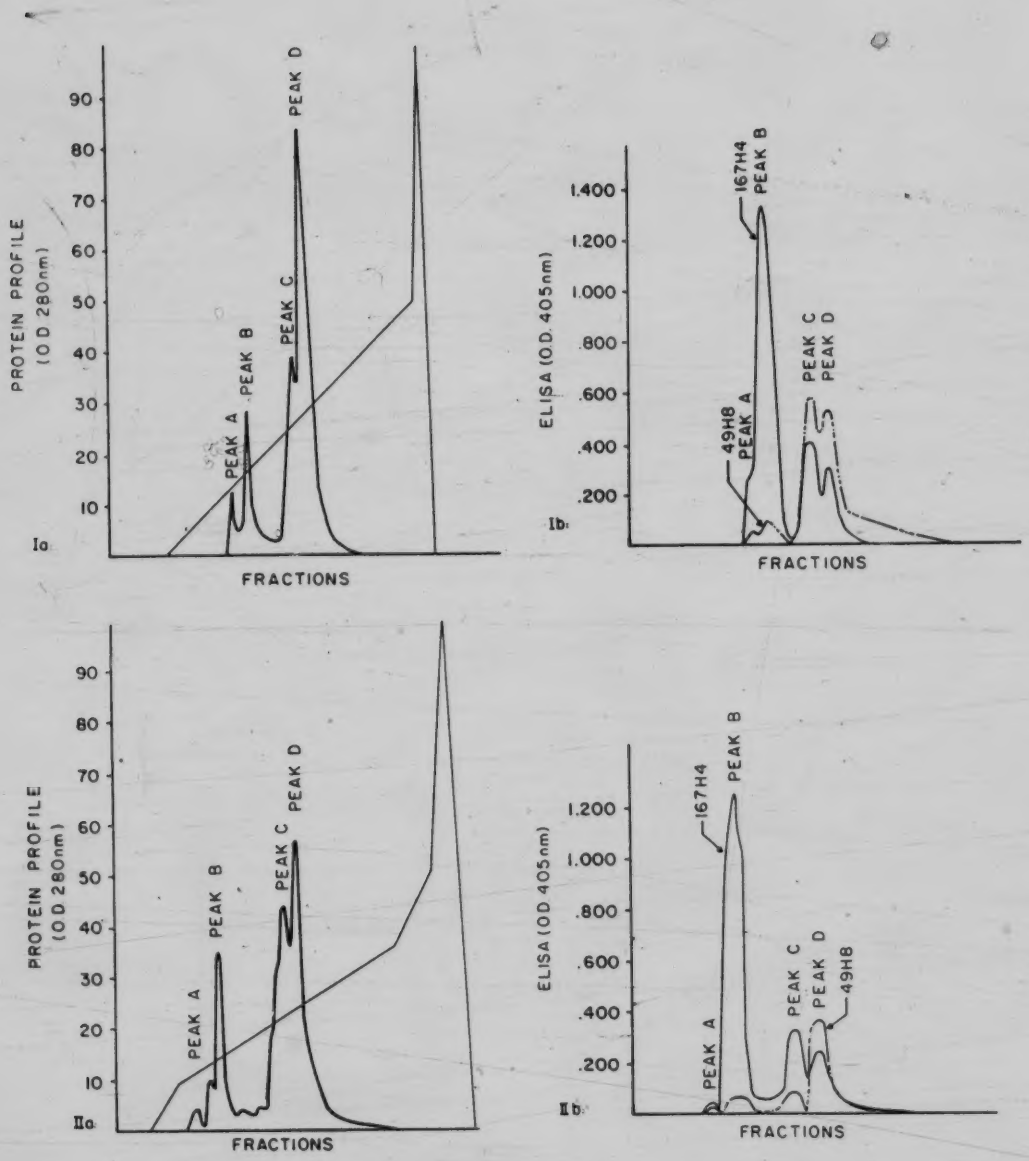


Figure 18. Purification of HTP Fractions by FPLC-Mono-Q.

For the FPLC chromatography (Pharmacia), Buffer A was a 20 mM Tris-HCl solution (pH 7.7) and Buffer B was the same but contained 58.44 g of NaCl per litre. Ib and IIb are the corresponding ELISA results for the fractions collected in Ia and IIa, respectively, which pertain to the "AFPr Filtrate" (I) or "AFPr Eluate" (II) of Figure 17, respectively. Note that the results are similar for both the AFPr Filtrate and Eluate yielding a peak B with the highest reactivity with the 167H.4 MAb.

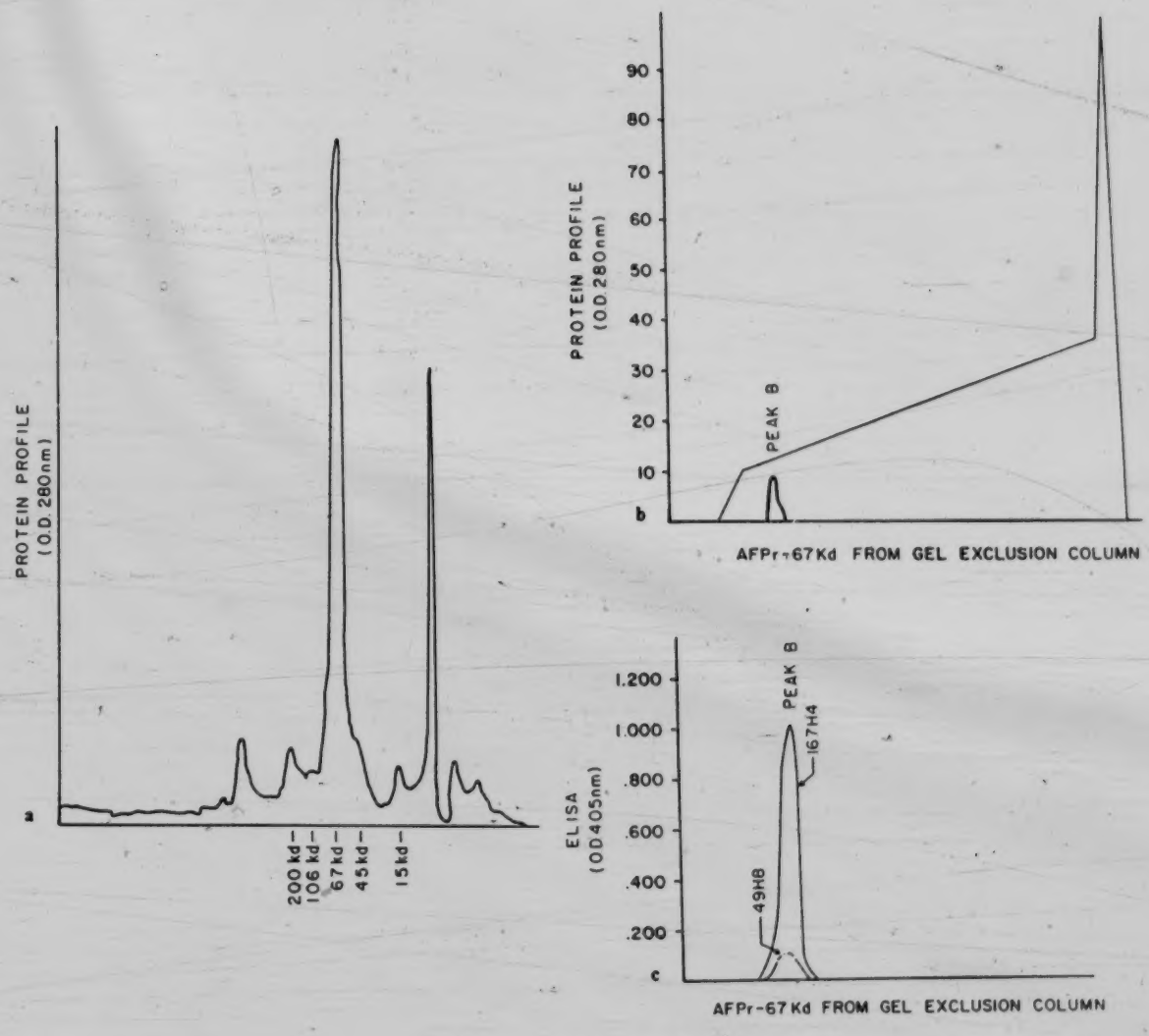


Figure 19. Purification of AFPr Eluate (HTP Fraction) by HPLC-Gel Filtration and Confirmation that Peak B contains a 67 kd Moiety Immunoreactive with 167H.4.

An aliquot of the HTP derived "AFPr Eluate" was run on a Bio-sil TSK column (Bio-Rad, 0.5 to 400 kd resolution) by HPLC (Pharmacia) in PBS (Figure 19a). A narrow fraction corresponding to the 67 kd major peak was then chromatographed by FPLC-Mono-Q (Figure 19b) and this was tested by ELISA (Figure 19c). Results show that a 67 kd major peak derived from the HTP fraction contains a 167H.4 reactive protein which shows a peak B profile as was found in Figure 18 by FPLC-Mono-Q.

Figure 20. Western Blotting of Purified Peak B with the Rabbit Antiserum to Crude AFP/AFP-BP.

The western-blot was performed from a 10% SDS-PAGE gel under non-reducing (1) and reducing (2) conditions with the rabbit antisera made to the crude AFP/AFP-BP. The control (pre-serum) was negative when also tested at 1/300 dilution (data not shown). Under reducing conditions, the reaction was considerably less showing only a faint band which did not photograph well.

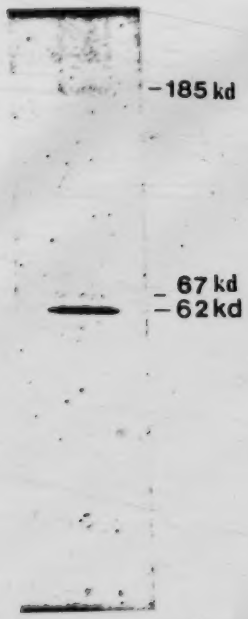


Figure 21. Peak B Purified From Breast Cancer Membranes Contains Mostly a 62 Kd Moiety but Also a 185 Kd Band by Silver Staining.

Peak B was purified from NP40 solubilized, pooled breast cancer membranes according to the procedure outlined in Figures 17-18 and was visualized by silver staining of 10-15% SDS-PAGE Phast-gels under reducing conditions. A major band of 62 kd was found and weak bands of 67 kd and 185 kd were also visualized.

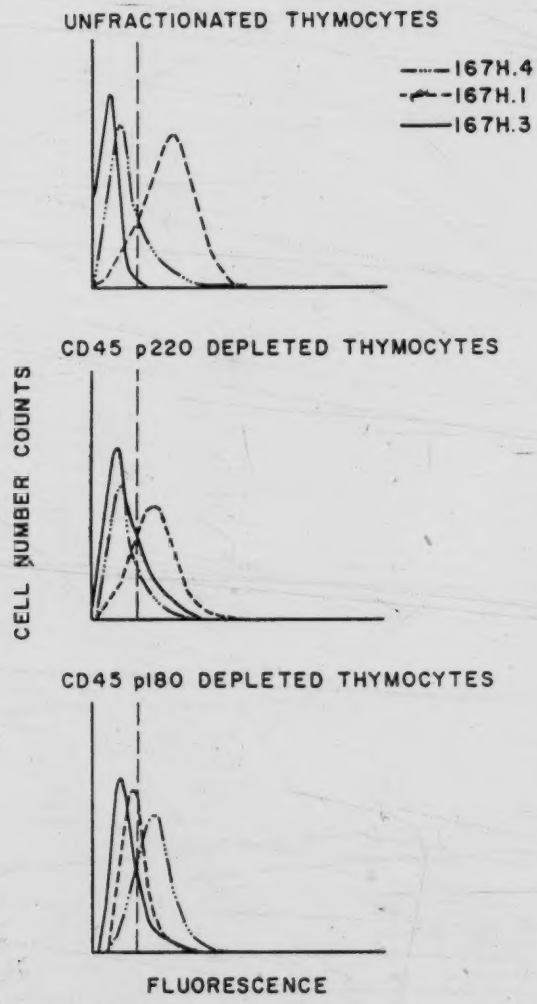


Figure 22. FACS Analysis on Human Thymocyte CD45 Isoform Subpopulations.

Human thymocytes were depleted of CD45RA (p220+) cells or CD45RO (p180+) cells according to the methods outlined in Materials and Methods and were stained by the indirect technique for expression of the 167H.1 or 167H.4 reactive isoforms. 167H.3 is an IgM MAb of unknown specificity but which reacts with subpopulations of peripheral blood mononuclear cells (Table 23). The 167H.1 MAb preferentially reacts with CD45RO (p180+) cells, whereas the 167H.4 MAb preferentially reacts with CD45RA (p220+) cells. Representative of 3 experiments. Results supplied by Dr. Linda Pilarski.

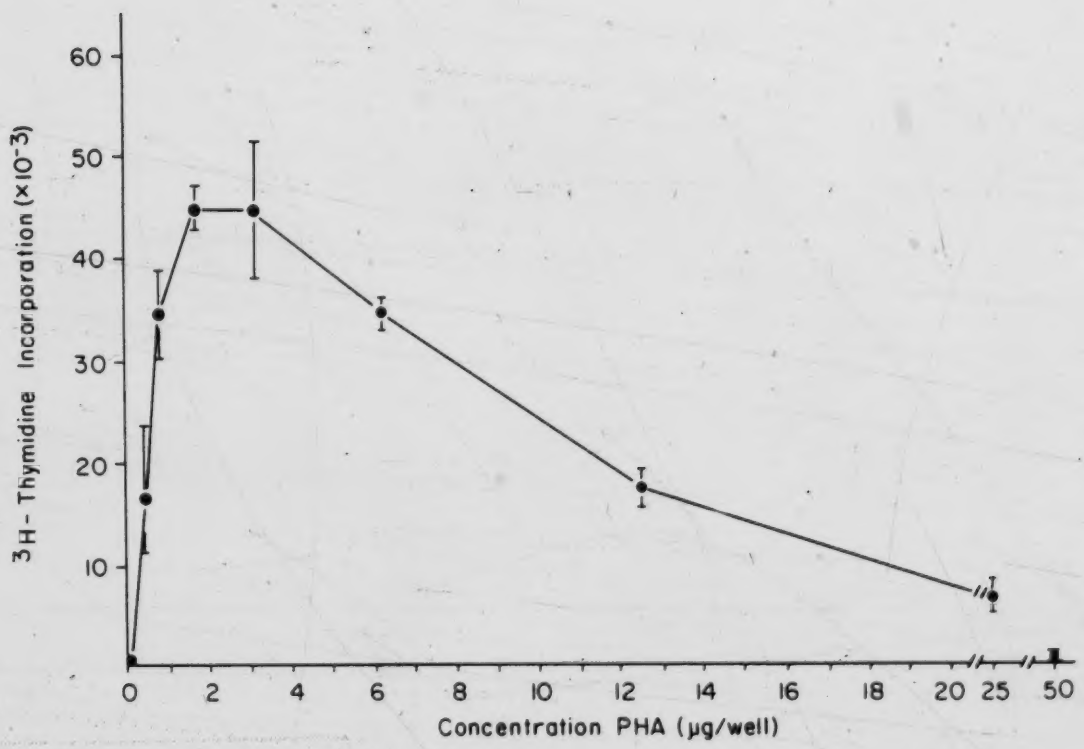


Figure 23. The 72 Hour PHA Response of Human PBMC: Dose Response.

Results are expressed as the means and standard errors of quadruplicates of ^{3}H-thymidine incorporation (cpm x 10^{-3}).

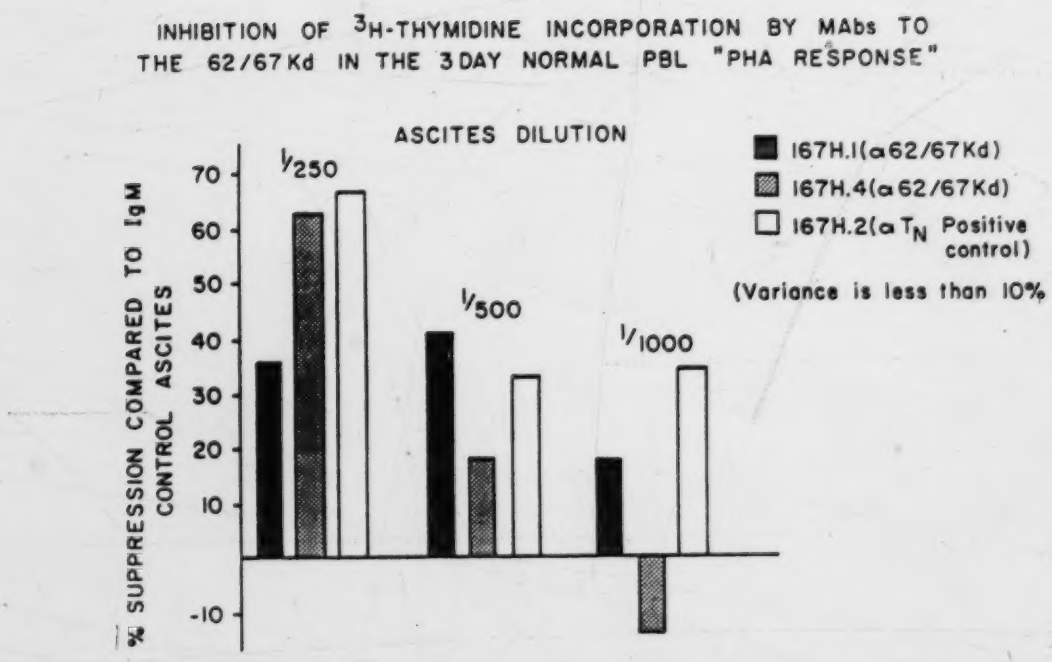


Figure 24. Anti-AFP-BP MAbs Inhibit the PBMC PHA Response.

Results are expressed as the means of the percent suppression of the indicated ascites (in duplicates) normalized to a control IgM MAb, 167H.3 analysed by ^{3}H-thymidine incorporation in response to 2 ug/well of PHA in 10% FCS-RPMI. Variance was less than 10%. Control wells without added antibody gave 69,737 (±6810) cpm of thymidine incorporation. The 167H.3 MAb reacts with human PBMC (Table 23).

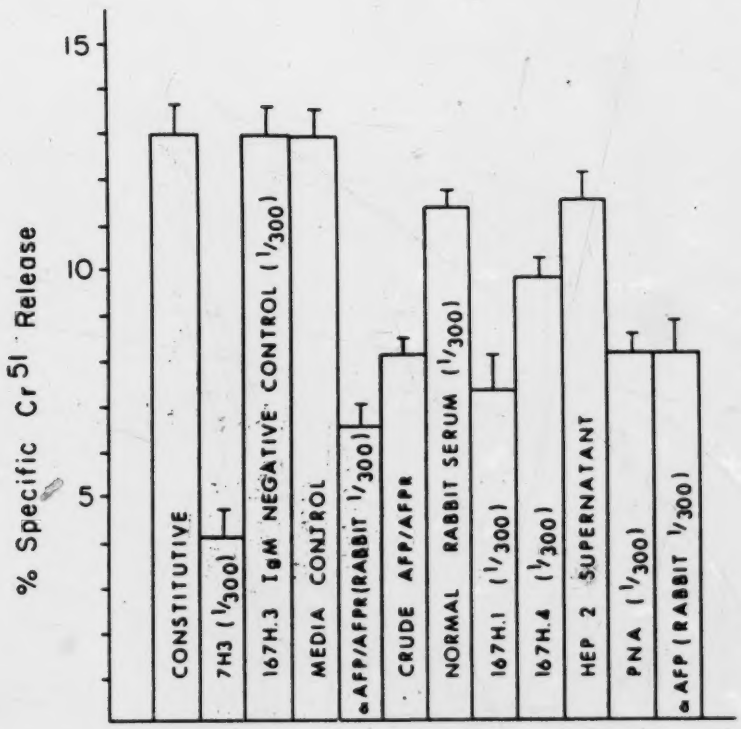


Figure 25. Anti-AFP-BP MAbs Inhibit the Constitutive Adherent Cell Tumoricidal Activity of PBMC's.

Results are expressed as the means and standard errors of quadruplicates and % chromium release was calculated according to the Materials and Methods. 7H.3 is an anti-Class II MHC MAb, and PNA was a 1/300 dilution of a 1 mg/ml solution. The anti-AFP (rabbit) is a commercial antiserum. The assay was performed in 5% FCS-RPMI and is an 18 hour assay.

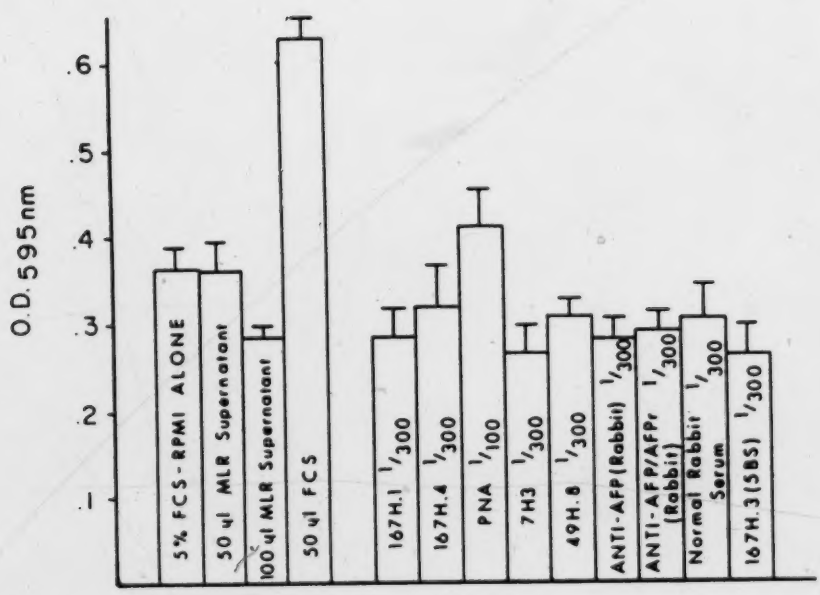


Figure 26. No Evidence for Non-specific Cytotoxicity of Adherent Cells by Treatment with Various Proteins/Abs by 48 Hours As Measured by the MTT Assay.

Results were expressed as the means and standard errors of quadruplicates and were performed in the presence of 5% FCS. Ascites or antisera were diluted 1/300. PNA was 1/300 dilution of a 1 mg/ml solution. By MTT, there was no evidence that any of the added proteins were cytotoxic to the adherent cells.

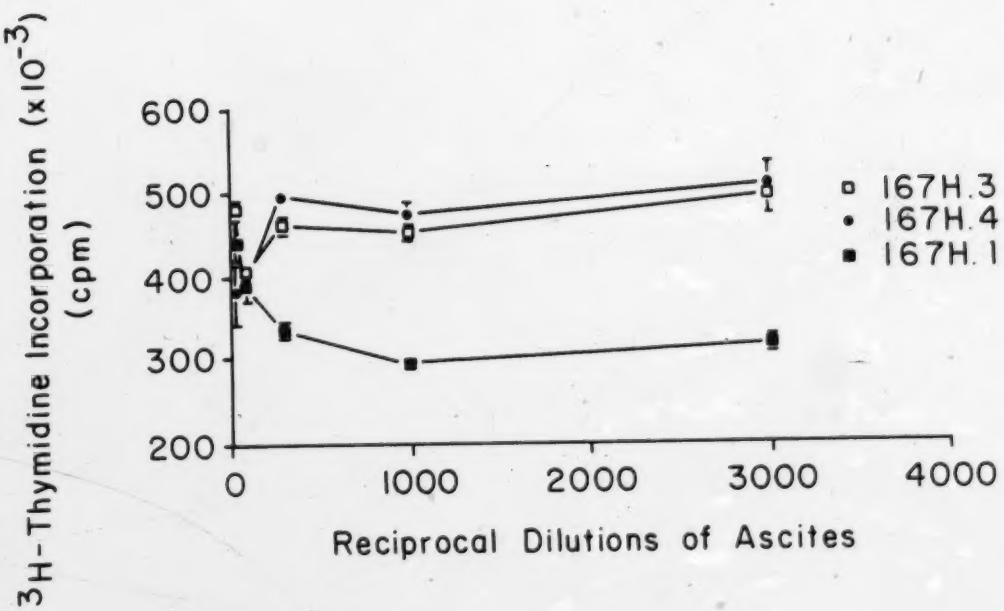


Figure 27. The 167H.1 MAb Inhibits LoVo Cell Proliferation in 10% FCS (48 Hours).

Results are expressed as the means and standard errors of cpm of thymidine incorporated by $1x10^5$ cells/well by 48 hours. The results show that the 167H.1 but not the 167H.4 or 167H.3 IgM ascites was able to inhibit the replication of DNA.

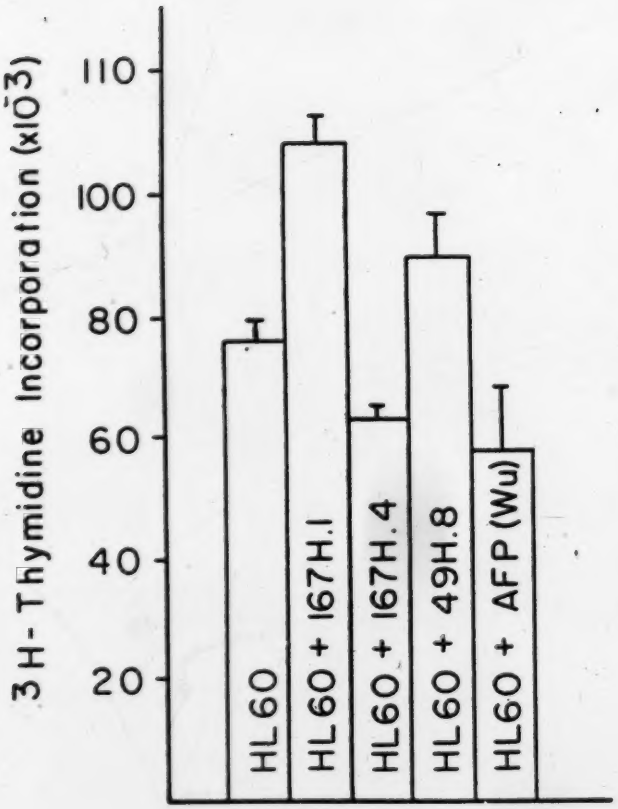


Figure 28. The 167H.1 MAb Enhances the Proliferation of HL-60 Cells Cultured in 1% Ultrasor at 48 Hours as Measured by ^{3}H-Thymidine Incorporation.

Results are expressed as the means and standard errors of quadruplicates (cpm x 10^{-3}). Purified proteins were used at 50 ug/ml. Similar results were obtained in 2% FCS-RPMI or by the MTT assay, wherein the 167H.1 MAb showed approximately 30% enhancement. AFP (Wu) is a commercially available ultrapure preparation of AFP but which has been denatured during the purification procedure.

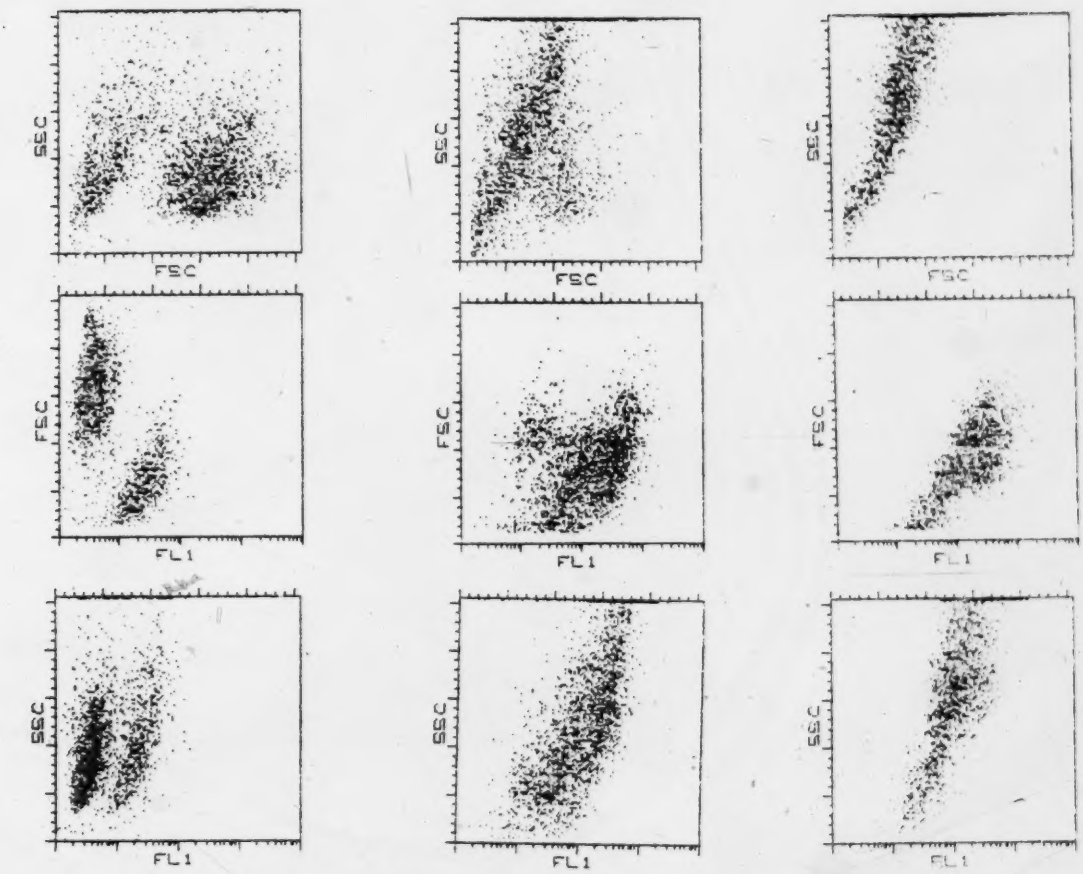


Figure 29. Cell Death in HL-60 Cells Cultured at Different Densities in 96 Well Plates.

HL-60 cells (passage number 3) were cultured at A) $1x10^4$; B) $1x10^5$; C) $1x10^6$ per 96 Linbro well for 96 hours in 5% FCS-RPMI (no phenol red) and then stained with 0.5 mg/ml FITC for 15 minutes at 37 C. After washing with cold PBS and fixation in 1% formalin-PBS, the cells were analysed by FACSCAN. Note that with an increase in cell density (number of cells plated per well; A,B,C), there are a higher percentage of cells with decreased size (FSC), increased granularity (SSC), and enhanced FITC uptake (FL1), which is typical of dying/dead cells. The results indicate that subcultures of HL-60 cells in 96 well Linbro plates is associated with the induction of death by 96 hours and that the relative levels of death induction is regulated at least in part by cell density related interactions.

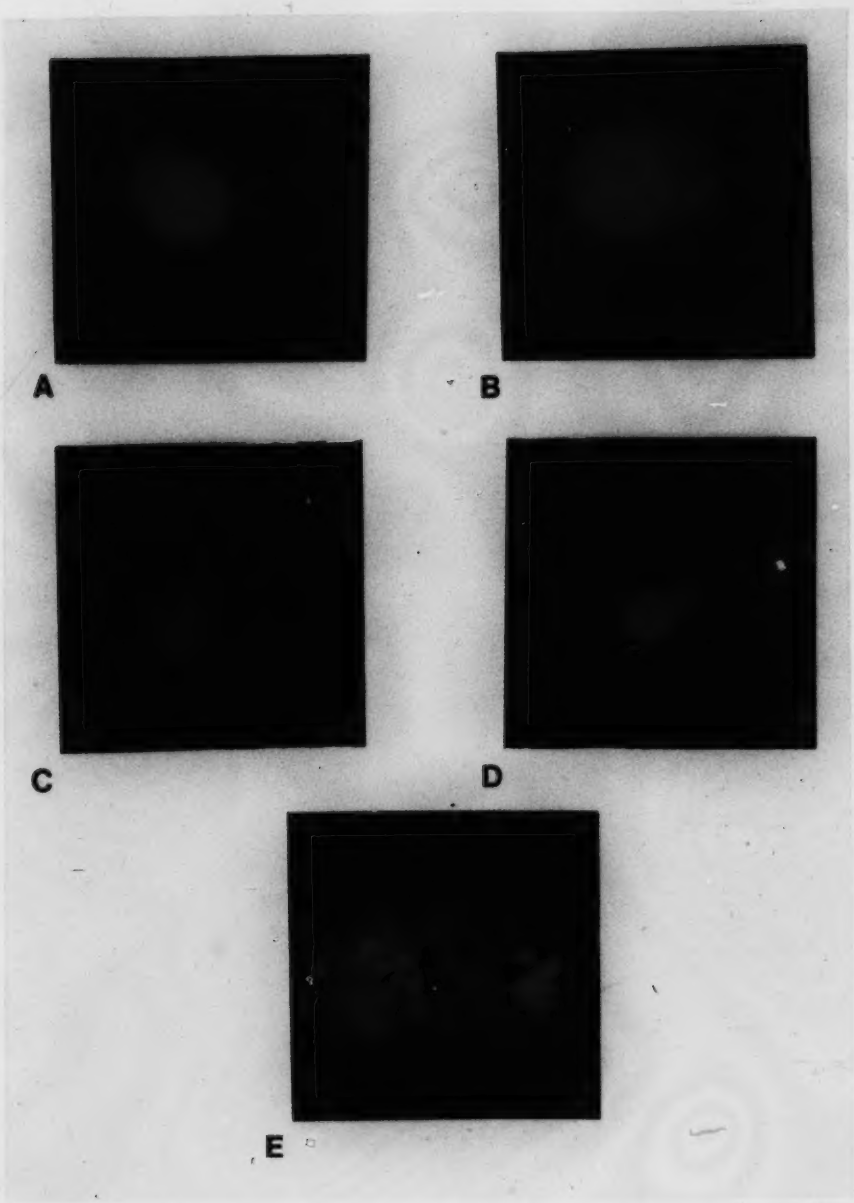
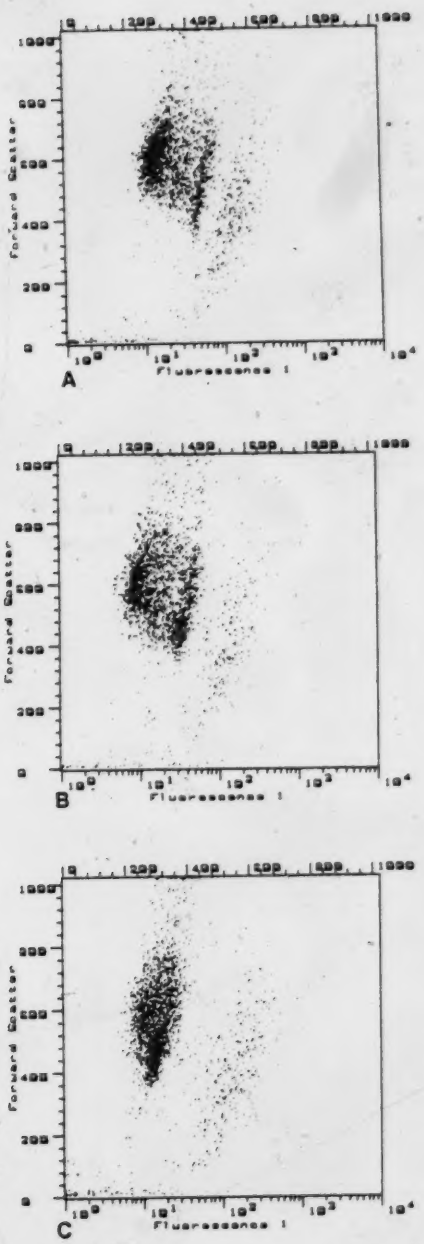


Figure 30. Morphological Staging of Death Induction of HL-60 Cells.

HL-60 cells were stained by FITC (0.5 mg/ml) for 15' at 37° C, washed and fixed before visualization under epi-fluorescence microscope. Progressive stages of death induction are shown A through E. Note the early blebbing in plates A-C, and the late blebbing in D, and the perforations and apoptotic bodies formation in plate E. Control viable HL-60 cells did not exhibit FITC uptake as evidenced under the microscope.



Control IgM MAb

167H.4 MAb

167H.1 MAb

Figure 31. Dot Plot Analysis of FITC Staining (FL-1) Versus Forward Scatter of HL-60 Cells at Passage 3 at 84 Hours.

HL-60 cells were cultured at $1x10^5$ cells per well in 96 well Linbro plates in 5% FCS-RPMI (no phenol red) and harvested at 84 hours. The results show that the 167H.1 MAb blocks FITC uptake relative to the 167H.4 or control MAb (NSC C1 20). All MAbs were used at 10 ug/ml.

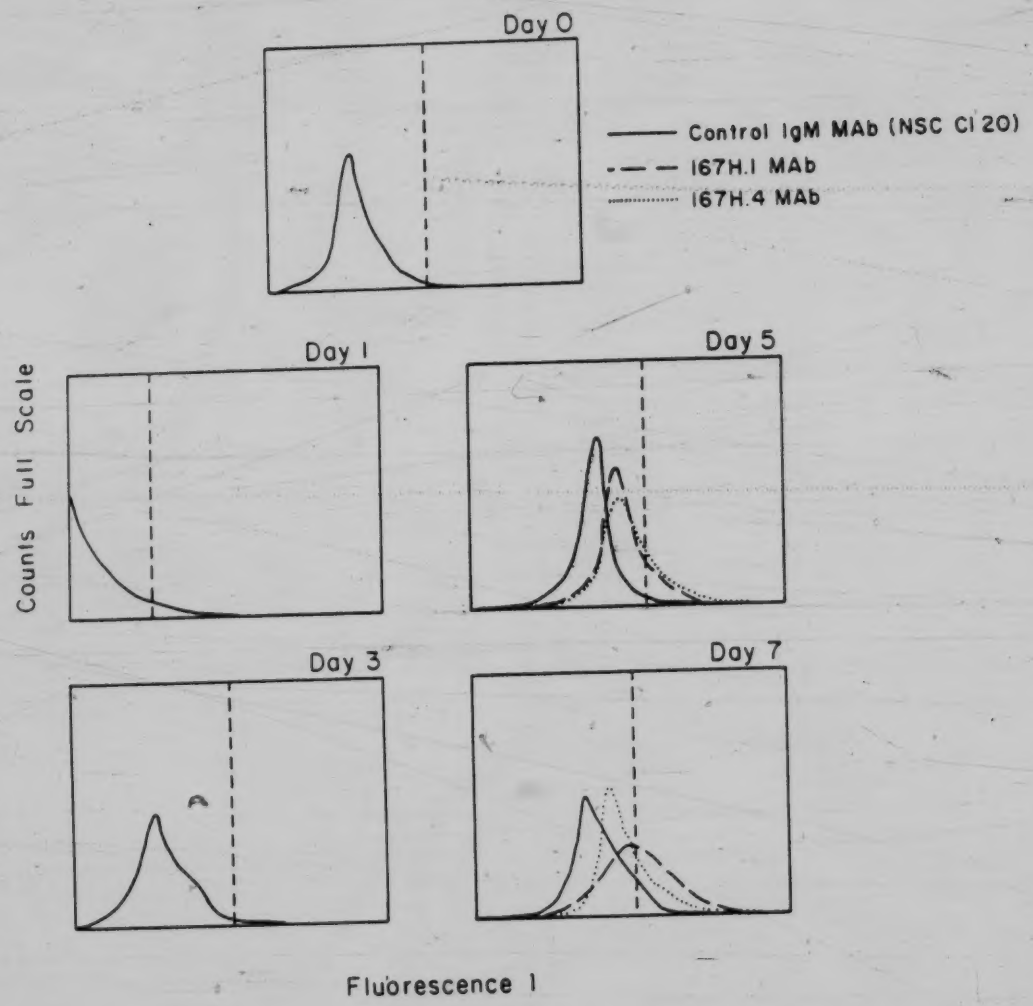


Figure 32. Expression of the AFP-BP on Cultured Multinegative Human Thymocytes does not Occur Until Approximately Day 5 of Culture.

Multinegative human thymocytes ($CD3^-$, $CD4^-$, $CD8^-$) were isolated according to the depletion procedure detailed in Materials and Methods. The cells were plated at $1x10^5$ cells/well (96 well Linbro plates) in 5% FCS-RPMI (no phenol red) and analysed for expression of the AFP-BP by an indirect FACS method. Results are representative of two independent experiments. Fluorescence 1 = FITC.

On days 0, 1, and 3 the 167H.1 and 167H.4 profiles were identical to the control IgM MAb (NSC CI 20), indicating the AFP-BP is not expressed on early multinegative thymocytes. By day 5, 17.1%, 24.2%, and 3.7% of the cells were positive for the 167H.1, 167H.4 and IgM control MAbs, respectively. By day 7, 68.65%, 29.0%, and 11.0% of the cells were positive for the 167H.1, 167H.4 and control IgM MAbs, respectively.

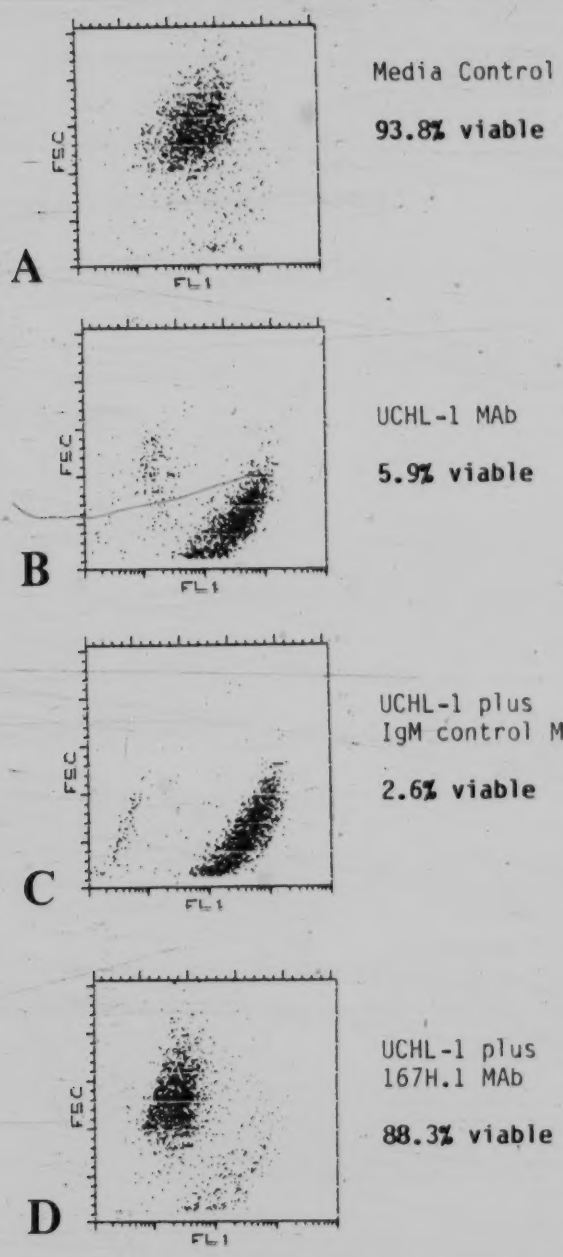


Figure 33. PCD of HL-60 Cells Induced by the UCHL-1 (anti-CD45RO p180) MAb is Blocked by the 167H.1 MAb.

Passage 13 HL-60 cells were cultured in 5% FCS-RPMI at $1x10^5$ cells/well for 96 hours with (B,C,D) or without (A) 140 ug/ml of the UCHL-1 MAb then stained for viability with FITC. The results indicate that the 167H.1 IgM MAb (100 ug/ml) blocks death induction by the UCHL-1 MAb (anti-CD45RO). % viable refers to the % of cells which are larger (higher forward scatter) and less fluorescent (lower FL1). 98% of the death induced by the UCHL-1 MAb was blocked by pretreatment of HL-60 cells with the 167H.1 MAb. The IgM control MAb was NSC C1 20 also employed at 100 ug/ml.

Figure 34. Autoradiography of Proteins Phosphorylated by the 167H.1 MAb.

The in vivo protein kinase experiment was conducted according to the procedure outlined in Materials and Methods. Total is the unprecipitated ^{32}P labeled proteins of G361 human melanoma cells after a 20 minute triggering with either the 167H.1 or control IgM CH4 MAb. The 167H.1 MAb appears to specifically activate the phosphorylation of a band with a molecular weight around 200 kd, which was not activated by the the control MAb, although both had been immunoprecipitated by the rabbit anti-AFP/AFP-BP (IMM'PT). S refers to the radio-labeled standards and the film was developed for an exposure time of 18 hours. (See Table 38 for ratio of specific binding).

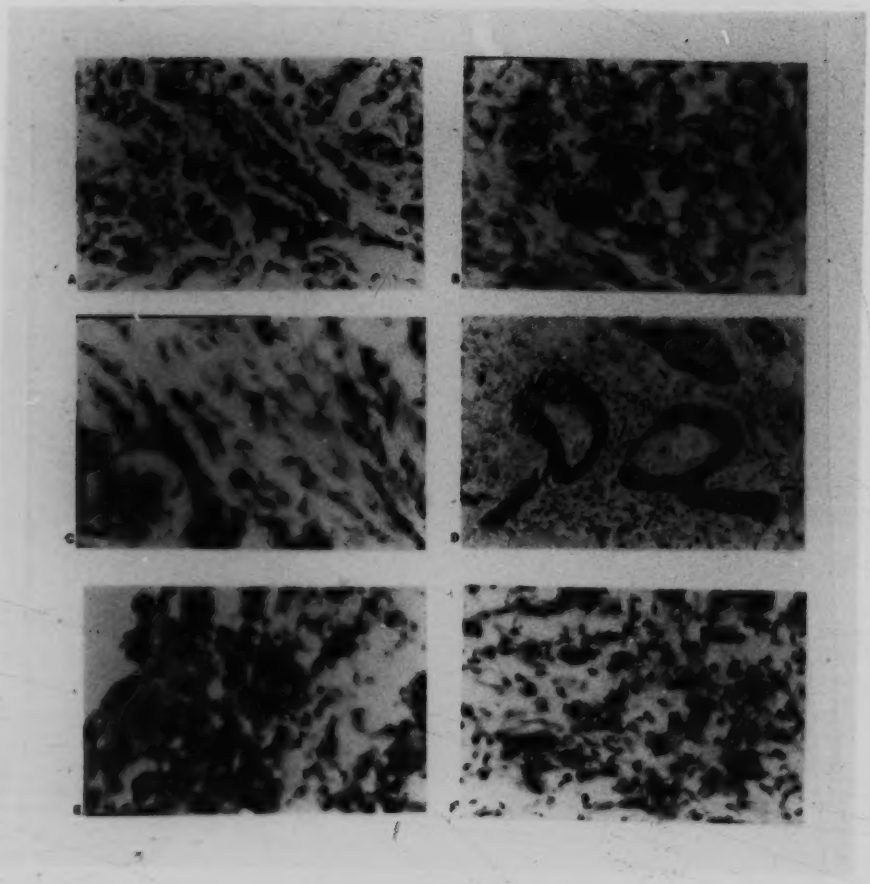


Plate 1. **Expression of the AFPr/AFP-BP on Malignant Tissues by the Immunoperoxidase Technique in Frozen Sections.** Brown staining represents positive reactions. A) Infiltrating Lobular Cancer of Breast, 167H.1. Note the homogenous and membrane accentuation of the staining. B) Moderately Differentiated Adenocarcinoma of Lung, 167H.4. Note the membrane accentuation. C) Poorly Differentiated Invasive Gastric Cancer, 167H.4. D) Metastatic, Well Differentiated Colonic Cancer, 167H.1. E) Keratinizing Squamous Carcinoma, 167H.4. F) Metastatic, Poorly Differentiated Colonic Cancer, 167H.4. Note the membrane accentuation.

Plate 3. **Expression of the AFPr/AFP-BP on Fetal Tissues by the Immunoperoxidase Technique on Frozen Sections.** A) Neural Crest Cells of Fetus, 167H.1. Note the homogenous staining without membrane accentuation. B) Placenta Showing Trophoblast Cells Positive, 167H.1.

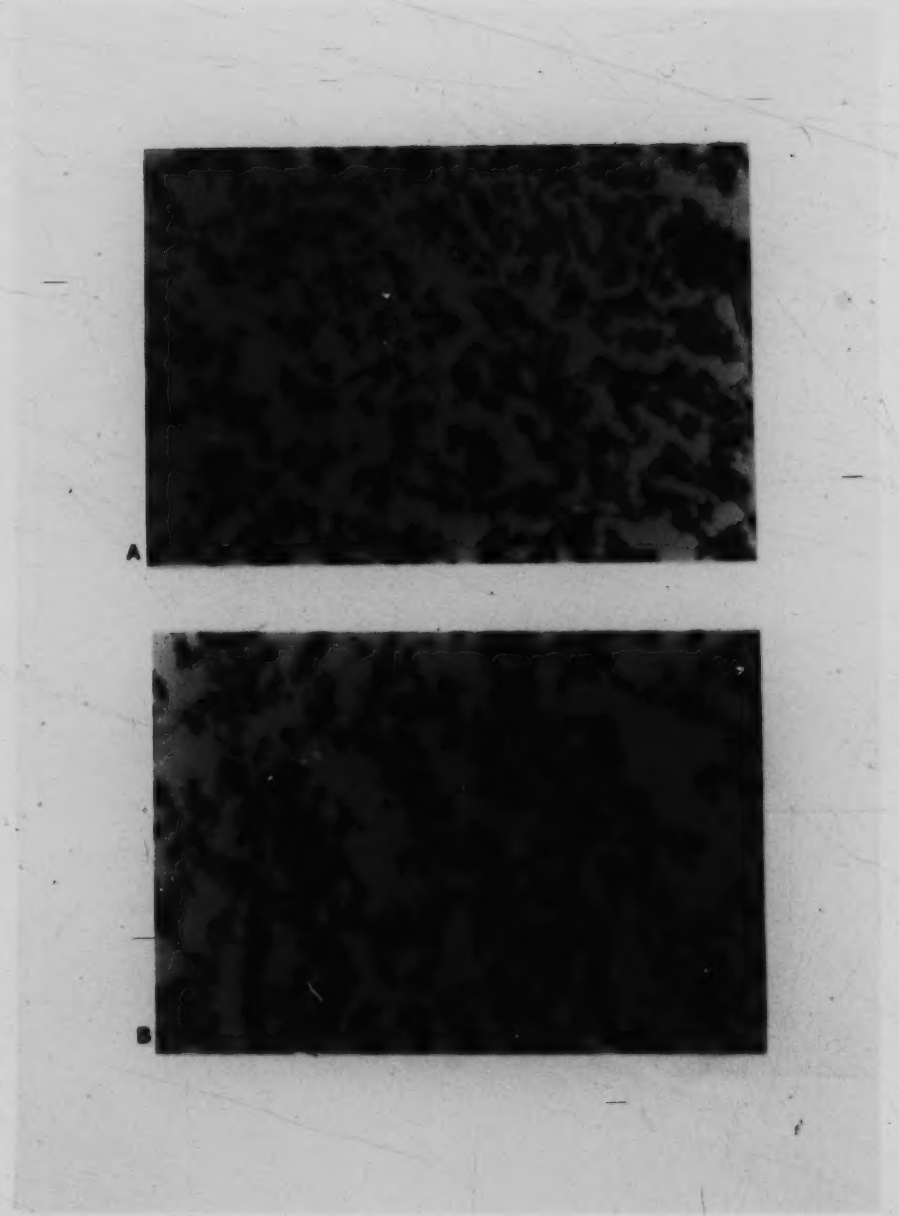


Plate 4. **Expression of the AFPr/AFP-BP on Central Nervous System Tissue by the Immunoperoxidase Technique on Frozen Sections.** A) Normal Brain, 167H.1. Neurons are positive as well as some glial cells. B) Normal Brain, 167H.4. Neurons and glial cells are positive.

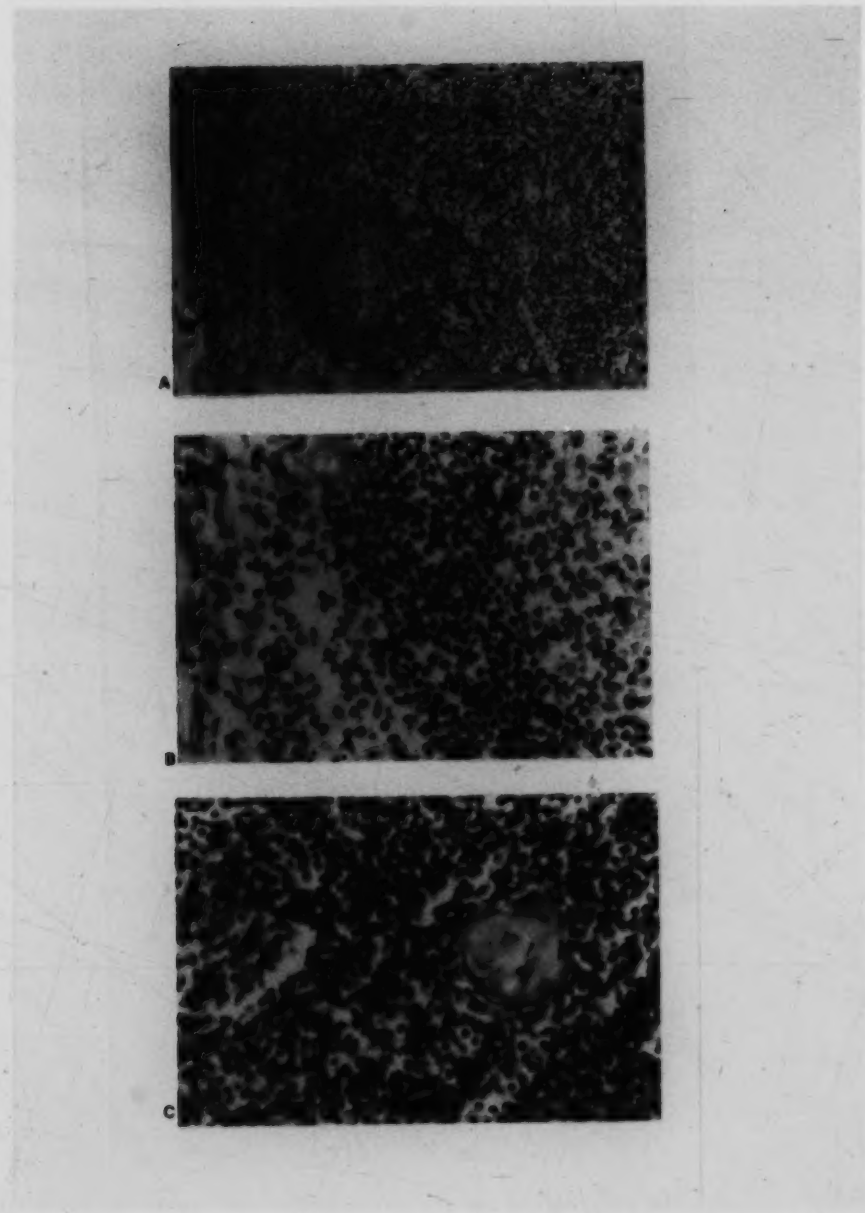


Plate 5. **Expression of the AFPr/AFP-BP on Cells of the Immune System by the Immunoperoxidase Technique on Frozen Sections.** A) Hyperplastic Lymph Node, 167H.1. The germinal center is negative, and macrophages are positive. B) Hyperplastic Lymph Node, 167H.1. Lymphocytes are negative but macrophages are positive. C) Thymus Medulla Showing Hassall's Corpusle, 167H.4. Dendritic cells and processes are positive, as is the Hassall's corpusle. Developing thymocytes appear to show variable, weak staining.

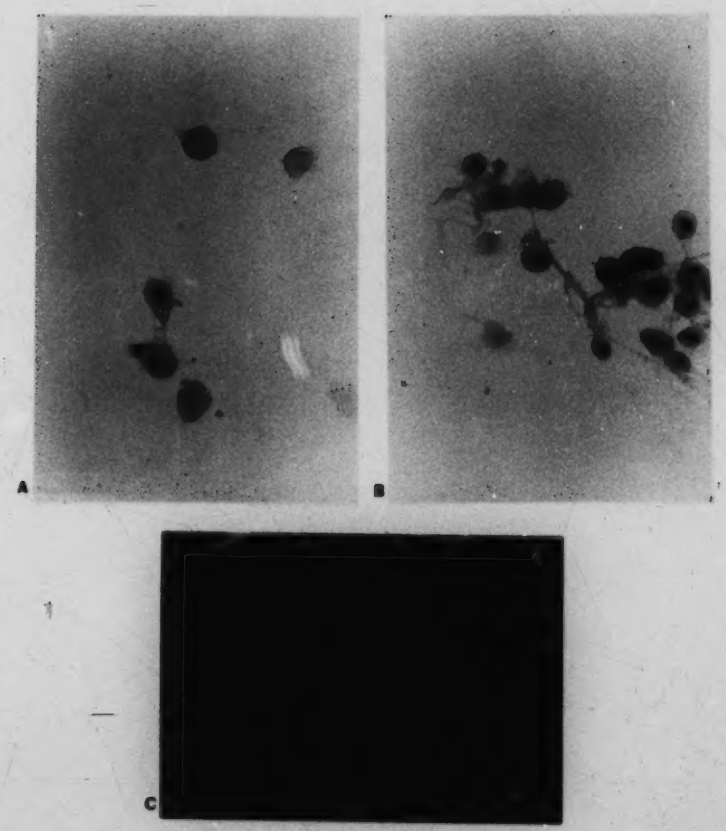


Plate 6. **Expression of the AFPr/AFP-BP on Cell Lines by the Immunoperoxidase Technique on Smears or by Immunofluorescence.** A) Smear, LoVo Cells, 167H.1. Note the membrane accentuation, but there is heterogeneity of expression of the 167H.1 reactive antigen. B) Smear, LoVo Cells, IgM Control MAb. C) Ichikawa Cells are 167H.4 Reactive by RITC Labeled Second Antibody and Fluorescence by Epi-fluorescence Microscope. Note the membrane accentuation and that some capping has occurred, as expected for a membrane associated functional receptor.

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